昭和32年7月9日　第三種郵便物認可
第45巻 第10号 昭和33年10月25日発行
(毎月1回25日発行)
The Journal of Biochemistry, published monthly by
the Japanese Biochemical Society, Tokyo, Japan.
Vol. 45, No. 10, October, 25, 1958

Vol. 45, No. 10 **October, 1958**

THE JOURNAL OF BIOCHEMISTRY

EDITED FOR THE JAPANESE BIOCHEMICAL SOCIETY

EDITORIAL BOARD

SHIRO AKABORI **SHIGERU AKAMATSU**
SHICHIRO AKIYA **FUJIO EGAMI**
SABURO FUNAHASHI **YOSOJI ITO**
KOOZOO KAZIRO **KATASHI MAKINO**
TOMOO MIWA **ZIRO NIKUNI**
NORIO SHIMAZONO **YUSUKE SUMIKI**
HIROSHI TAMIYA **ITARU WATANABE**
HARUHISA YOSHIKAWA

PUBLISHED MONTHLY

BY

THE JAPANESE BIOCHEMICAL SOCIETY

Tokyo University, Tokyo

AGENCIES

Charles E. Tuttle
Rutland, Vt.
U.S.A.

Maruzen Co., Ltd.
Tokyo
Japan

Santo Vanasio
Köln
Germany

PRINTED IN JAPAN

THE JOURNAL OF BIOCHEMISTRY was founded in 1922 by Prof. emeritus Dr. S. Kakiuchi and had been edited by him to volume 36 of the year of 1944. After the discontinuation during the War, this JOURNAL has been restarted by The Japanese Biochemical Society, following the serial number from volume 37 of the year of 1950.

This JOURNAL is collecting the original investigations on the subject in this country. It is published monthly, one volume per year.

Note to Contributors

All manuscripts should be carefully typewritten, triple-spaced, in English, French or German, and addressed to *The Editor, The Journal of Biochemistry, The Japanese Biochemical Society, c/o Departments of Biochemistry, Faculty of Medicine, Tokyo University, Bunkyo-ku, Tokyo, Japan.*

Authors receive 50 reprints of their papers as a rule at cost; additional copies can be prepared by the order on cost accounts. All orders for reprints should be markedly noted on the article paper or in a sheet of paper attached to it. On submitting the paper, it is preferable to attach the non-traced charts, besides original drawings, for circulation to Editors.

On request from authors to contribute to JOURNAL, the *Directions for preparation of manuscripts*, including styles of abbreviations of symbols, terms, units, and periodicals will be sent immediately.

Subscription

Subscription Price. ¥3,600 (U. S. $10.00) per volume, postage inclusive. Back issues are available for sale, from volume 1 up-to-date at ¥3,600 (U. S. $10.00) per volume, postage inclusive.

Remittances should be made preferably by check or draft payable to *The Japanese Biochemical Society, c/o Department of Biochemistry, Faculty of Medicine, Tokyo University Bunkyo-ku, Tokyo, Japan.*

Foreign subscription is treated by the agencies of *Charles E. Tuttle, Co.*, Rutland, Vermont, U.S.A., *Santo Vanasio*, Köln, Germany, and *Maruzen Co. Ltd.*, Chuo-ku Tokyo, Japan.

The Journal of Biochemistry, Vol. 45, No. 10, 1958

ENZYMATIC RESOLUTION OF RACEMIC AMINOACIDS*

VI. HYDROLYSIS OF VARIOUS *N*-ACYL DL-AMINO ACIDS BY MOLD ACYLASES

By KIMIYO MICHI ADN HARUMI TSUDA

(*From the Laboratory of Food and Nutrition of Japan Women's University, Tokyo*)

(Received for publication, November I2, 1657)

Greenstein *et al.* (*1–3*) have established a skillful method of preparation of optically-active amino acids by enzymatic resolution of the recemic amino acids. It depends upon the use of the renal acylase which is capable of acting asymmetrically only on the L-isomer of *N*-acyl DL-amino acids.

The present authors have been engaged in a series of stucies on the enzymatic resolution of racemic amino acids by using the mold acylase which acts only on the L-isomer of *N*-acyl DL-amino acids (*4–8*). It has been noted that the mold acylase is employed successfully in the resolution of various DL-amino acids, namely, DL-phenylalanine, DL-methionine, DL-valine, DL-glutamic acid, DL-leucine, DL-alanine, DL-tryptophan DL-lysine. This enzyme is especially more effective on the aromatic amino acids and the basic amino acids as compared with the renal acylase which is less effective on the aromatic amino acids, tyrosine and tryptophan, and the basic amino acids such as lysine.

Since *N*-acyl derivatives of various DL-amino acids have been prepared in addition to those which were so far resolved, specific activities of the mold acylase from two sources, *Penicillium* and *Aspergillus*, are measured for all these *N*-acyl amino acids and the data are summarized in the present paper.

EXPERIMENTAL

Substrates for Enzymatic Assay—Acetyl DL-phenylalanine, acetyl DL-methionine, chloroacetyl DL-valine and acetyl DL-glutamic acid are those which had employed previously (*5*, *6*).

The *N*-acyl derivatives of DL-amino acids which have been prepared in this laboratory are listed in Table I. These acyl DL-amino acids were prepared in the usual manner by the acetylation with acetic anhydride and sodium hydroxide.

---

* This report was presented before the Annual Meeting of Japanese Biochemical Society held on July 14, 1957.

Preparation of Enzyme: *Acylase of Penicillium*—The acylase of *Penicillium* was prepared from the cultures of *P. vinacious* on wheat bran (1.8 kg.) as described previously (7). The purified acylase was obtained by successive precipitation with ammonium sulfate of 0.6 saturation, acetone, and fractionation with rivanol, followed by elution from the rivanolenzyme complex. The final acetone precipitation yielded 87 mg. of dried enzyme. *Analysis*, N: 10.19 per cent.

TABLE I

Substrates

Acyl DL-amino acids	M.W.	m.p. (C)	N (%)	
			Found	Calcd
Acetyl DL-phenylalanine	207	146	6.73	6.74
Acetyl DL-tryptophan	246	G. B. I.1)		
Acetyl DL-tyrosine	223	90	6.13	6.27
Acetyl DL-methionine	191	112	7.38	7.32
Acetyl DL-alanine	131	G. B. I.1)		
Acetyl DL-valine	159	Donated2)		
Chloroacetyl DL-valine	194	130	7.41	7.20
Acetyl DL-isoleucine	173	149	8.12	8.10
Acetyl DL-leucine	173	G. B. I.1)		
Chloroacetyl DL.serine	182	Donated2)		
Chloroacetyl DL-threonine	196	Donated2)		
Acetyl DL-aspartic acid·Na·H_2O	215	146	6.56	6.52
Acetyl DL-glutamic acid	189	183	7.38	7.41
Acetyl DL-histidine·H_2O	215	150	19.41	19.53
Acetyl DL-arginine·$2H_2O$	252	Donated2)		
Diacetyl DL-lysine	230	141	11.87	12.10
ε-Benzoyl-α-acetyl DL-lysine	292	145	9.45	9.58

1) Purchased from General Biochemical, U.S.A.

2) Kindly donated by Dr. Greenstein of National Institutes of Health, U.S.A.

Acylas os Aspergillus—It was prepared from Takadiastase. Fifty g. of Takadiastase (Sankyo Co.) were dissolved in 600 ml. of water and added with ammonium sulfate to 0.6 saturation. The resulting precipitate which was separated by centrifugation was dissolved in water and dialyzed against distilled water overnight. The content of dialysis sack was treated with 2 per cent rivanol solution to complete precipitation of enzyme-rivanol complex. The brown viscous precipitate was separated by centrifugation. The precipitate was eluted several times each with 10 ml. of 0.5 *M* McIlvaine buffer (pH 6.2) as in the case of *Penicillium* acylase. The combined eluate was treated twice each with three and two g. of acid clay to remove the pigment. Almost colorless eluate was saturated with ammonium sulfate to salt out enzyme protein in an ice bath. The separated precipitate was centrifuged and dissolved in a small volume of water. The enzyme was precipitated again from the above solution by the addition of acetone.

The actone precipitate was dried over P_2O_5 and weighed 373 mg. *Analysis*, N: 12.32 per cent.

Enzymatic Assay—The acylase activity was estimated by the method which was described previously (*7*). The pH optima for each substrate were at first measured and the specific activity was expressed in terms of μM substrate hydrolyzed per hour per mg. N.

Effect of oobaltous ion on enzyme activity for various acyl amino acids was estimated. Aqueous solution of cobaltous sulfate was used at $10^{-3.7}$ molar concentration for each substrate.

RESULTS AND DISCUSSION

The acylase activities of the enzyme preparations from *Penicillium* and *Aspergillus* on various *N*-acyl DL-amino acids were investigated. The specific activities of both purified acylase were measured for eighteen acyl DL-amino acids.

TABLE II

Hydrolysis of Various N-Acyl DL-*Amino Acids with the Mold Acylases and Effect of Cobaltous Ion on Acylase Activity*

Substrates	Optimum pH	Acylase activity1)					
		of Penicillium acylase			of Aspergillus acylase		
		No Co^{++} μM/N *mg.*	Activation by Co^{++} μM/N *mg.*	%	No Co^{++} μM/N *mg.*	Activation by Co^{++} μM/N *mg.*	%
Acetyl DL-tyrosine	7.2	2277	2551	12	254	281	11
Acetyl DL-phenylalanine	7.8	2022	3121	54	212	309	46
Acetyl DL-tryptophan	7.8	1915	2580	35	192	277	44
Acetyl DL-methionine	7.4	1687	2445	45	159	240	50
Acetyl DL-alanine	7.8	1670	2090	26	148	181	22
Acetyl DL-isoleucine	7.6	580	745	29	81	107	33
Acetyl DL-leacine	7.8	565	1190	110	75	140	88
Acetyl DL-valine	7.4	445	785	76	43	75	74
Chloroacetyl DL-valine	7.6	703	540	−24	68	52	−24
Chloroacetyl DL-serine	7.8	1480	1965	33	204	255	25
Chloroacetyl DL-threonine	7.4	308	333	8	34	39	14
Acetyl DL-arginine	7.8	2240	2780	24	166	219	32
ε-Benzoyl-α-acetyl DL-lysine	7.6	2460	3110	27	224	307	37
Diacetyl DL-lysine	7.8	1493	2290	53	145	220	51
Acetyl DL-histidine	7.8	388	595	54	27	46	68
Acetyl DL-glutamic acid	5.6	1635	1465	−10	75	77	3
Acetyl DL-aspartic acid	5.4 2)	159	172	8	19	21	10
Acetyl D-phenylalanine		0	0		0	0	

1) Expressed in terms of μM substrate hydrolyzed per hour per mg. N of enzyme.

2) Optimum pH of acetyl DL-aspartic acid is 5.4 or less.

As shown in Table II, pH optima were found to range from 7.2 to 7.8 for acetyl derivatives of α-amino-monocarboxylic acid and from 5.4 to 5.6 for those of α-amino-dicarboxylic acid. The specific activities were expressed in terms of micromoles of liberated amino acid per hour per mg. N of enzyme preparation at each optimum pH. The data in Table II indicate that the acyl derivatives of aromatic amino acids, namely, tyrosine, phenyl-alanine and tryptophan, and those of basic of amino acids, namely, arginine and lysine, are hydrolyzed by the acylases of *Penicillium* and *Aspergillus* with almost equall ease. Those of methionine, alanine, and glutamic acid are also susceptible substrates. By both of these mold acylases acyl derivatives of the other aliphatic amino acid were hydrolyzed less easily and at progressively decreasing rates in the following order: acetyl DL-isoleucine>acetyl DL-leucine>acetyl DL-valine >acetyl DL-aspartic acid. Hydroxyamino acids, such as threonine and serine were assayed for chloroacetyl derivatives. Chloroacetyl DL-serine was found to be a susceptible substrate while chloroacetyl DL-threonine was rather resistant to the mole acylase.

Table II also indicates the effect of cobaltous ion on acylase activity. The effect was tested at $10^{-3.7}$ molar concentration where this salt had been found previously to accelerate most the hydrolysis of acetyl DL-phenylalanine (*7*). At this concentration, cobaltous ion on accelerated the activity of acylase on various other acyl DL-amino acids, especially, acetyl DL-phenylalanine, acetyl DL-methionine, diacetyl DL-lysine by 50 per cent, acetyl DL-varine by 75 per cent and acetyl DL-leucine by over 100 per cent. As exceptions, however, chloroacetyl DL-valine and acetyl DL-glutamic acid were found to be inhibited by cobaltous ion.

Another purpose of this research was an attempt to see whether or not the *Penicillium* acylase and the *Aspergillus* acylase are the same enzyme. As shown in Table II, no appreciable difference was found in the activity pattern for various *N*-acyl amino acids between acylases of *Penicillium* and *Aspergillus*, except the behavior of acetyl DL-arginine and acetyl DL-glutamic acid. The purified *Penicillium* acylase was much more active on acetyl DL-arginine and acetyl DL-glutamic acid than was the enzyme from Takadiastase. It was also found that the hydrolysis of most acyl amino acids by these two enzymes was activated by CO^{++} ion to about the same extent. On the contrary the enzymatic hydrolysis of chloroacetyl DL-valine was inhibited by Co^{++} ion. Again in this case the degree of inhibition was nearly the same for the acylase of *Penicillium* and that *Aspergillus*. All these findings would indicate that the mold acylases from two different sources may probably be the same enzyme.

SUMMARY

1. The acylase activities of the enzyme preparations from *Penicillum* and *Aspergillus* on various *N*-acyl DL-amino acids were measured.

2. Of these acyl derivatives of some fifteen DL-amino acids tested, the acetyl derivatives of aromatic amino acids, namely, tyrosine, phenylalanine and tryptophan and those of basic amino acids, such as arginine and lysine were hydrolyzed by the both mold acylases with almost equall ease.

3. The acetyl derivatives of aliphatic amino acids, such as methionine, alanine and glutamic acid were also susceptible substrates, although the rate of hydrolysis was somewhat lower than those above.

4. Hydroxy amino acids, such as threonine and serine, were tested in form of chloroacetyl derivatives. It was found that chloroacetyl DL-threonine was rather resistant, whereas chloroacetyl DL-serine was susceptible to the acylase.

5. The acetyl derivatives of other amino acids, such as isoleucine, leucine, valine and histidine, were hydrolyzed less easily. Acetyl DL-aspartic acid was the most resistant to the acylase.

6. Co^{++} ion at a concentration of $10^{-3,7} M$ accelerates the enzyme activity on various acyl DL-amino acids by about 10 to 100 per cent, with the exception that the hydrolysis of chloroacetyl DL-valine and acetyl DL-glutamic acid is inhibited.

7. Acylases from *Penicillium* and *Aspergillus* are probably the same enzyme.

The authors are grateful to Dr. J. Greenstein of National Institutes of Health, U.S.A. for generous supply of some of the acyl amino acids, to Dr. S. Tatsuoka of Takeda Pharmaceutical Industries for supplying DL-lysiee, and to Miss Isobe and Mrs. Sugimoto for performing the microanalyses. The research was aided by the Ajinomoto Company.

REFERENCES

(1) Birnbaum, S.M., Levintow, L., Kingsley, R.B., and Greenstein, J.P., *J. Biol. Chem.*, **194**, 455 (1952)
(2) Rao, K.R., Birnbaum, S.M., Kingsley, R.B., and Greenstein, J.P., *J. Biol. Chem.*, **198**, 507 (1952)
(3) Price, V.E., Gilbert, J.B., and ,Greenstein J.P., *J. Biol. Chem.* **179**, 1169 (1949)
(4) Michi, K. and Nonaka, H., *J. Agr. Chem. Soc. Japan*, **28**, 343 (1954)
(5) Michi, K. and Nonaka, H., *J. Agr. Chew. Soc. Japan*, **28**, 346 (1954)
(6) Michi, K. and Nonaka, H., *Bull. Agr. Chem. Soc. Japan*, **19**, 153 (1955)
(7) Michi, K. and Tsuda, H., *Bull. Agr. Chem. Soc. Japan*, **21**, 18 (1957)
(8) Michi, K. and Tsuda, H., *Bull. Agr. Chem. Soc. Japan*, **21**, 235 (1957)

Digitized by the Internet Archive
in 2024

https://archive.org/details/journal-of-biochemistry_1958-10_45_10

The Journal of Biochemistry, Vol. 45, No. 10, 1958

LOCALIZATION OF SUCCINOXICASE IN BACILLUS MEGATERIUM KM

By DAISUKE NAKADA, KEIICHI NOZU AND MASAMI KONDO

(*From the Department of Bacteriology, Research Institute for Microbial Disease, Osaka University, Osaka*)

(Received for publication, November 29, 1957)

It has been pointed out by many workers that the terminal respiratory enzymes and cytochromes are localized to the particulate fraction sedimentable by ultracentrifugation after mechanical disruption yf the bacterial cells. But the site in a native cell where these particles are derived from is yet uncertain.

On the other hand, Weibull (*1*, *2*) has shown that cytoplasmic membrane-like bodies termed "ghosts" are obtainable after osmotic lysis of protoplasts of *Bacillus megaterium* KM. The almost exclusive association of the bacterial cytochromes with the ghosts was also confirmed in his experiments. Subsequently, Weibull (*3*) has reported an evidence that the ghosts is the cytoplasmic membrane, perhaps in a denatured form.

In the present study are reported some experiments designed to clarify the native site of the intracellular particles possessing succinoxidase activity and cytochrome components, and considerations that these particles may be derived from the ghosts by mechanical disruption of the bacterial cells.

MATERIALS AND METHODS

Bacillus megaterium KM was grown at 35° with aeration by shaking. The medium, pH 7.0, contained 2 per cent Bacto-pepton, 0.5 per cent NaCl and 0.5 per cent glucose. Cells were harvested by centrifugation at the end of the exponential growth phase, *i.e.*, after 4 hours incubation under the present conditions.

Protoplasts were prepared as follows. Cells harvested were washed twice with C medium (*4*) consisting of NH_4Cl, 2 g ; KH_2PO_4, 6 g ; NaCl, 3 g ; $MgCl_2$, 40 mg., Na_2SO_4, 110 mg. ; distilled water, 1,000 ml. ; pH 6.9, and suspended in the same medium containing 10 per cent polyethylene glycol (Carbowax 4000). Usually 30 to 60 mg. nitrogen of cells and 10 to 20 ml. of suspending medium were employed in a single batch. Lysozyme was added to 0.05 per cent in a final concentration. Protoplast formation was complete after about 30 minutes at room temperature. Protoplasts were sedimented by centrifugation at 8000 r. p. m. for 10 minutes in the cold, and resuspended in C medium containing 10 per cent carbowax. Supernant was discarded.

For the preparation of ghosts, the protoplasts centrifuged down were resuspended in a hypotonic C medium not containing Carbowox. Immediately osmotic lysis of the protoplasts occured and after 2 minutes of vigorous agitation 50 per cent carbowax was added to make a final concentration of 10 per cent. To decrease the viscosity of the lysate, a small amount of deoxyribonuclease (final 1 μg. per ml.) was added. After viscosity was decreased, the lysate was centrifuged at 12,000 r. p. m. (14,500×g) for 20 minutes. The resulting sediment was resuspended in a C medium with 10 per cent carbowax, and used at the ghost fraction. Supernatant was also used in the following experiments. All operations described above were carried out at 0 to 5°.

Mechanical disruption of bacterial cells and ghosts was conducted by the use of a 9 kc Raytheon sonic oscillator or by grinding with glass powder. After disruption, differential centrifugation was performed in a Spinco L preparative ultracentrifuge. C medium with 10 per cent carbowax was used as a suspending medium in all subsequent operations. Large cell debris or glass powder removed by centrifugation at 4,000 r. p. m. for 10 minutes. The resulting crude extract was then centrifuged at 12,000 r. p. m. (14,500×g) for 20 minutes, and separated into two fractions, the supernatant (S_1) and precipitate (P_1). The S_1 fraction was further centrifuged at 35,000 r. p. m. (approximately 100,000×g) for 45 minutes, and S_2 and P_2 fractions were obtained.

Enzymatic activities were measured with a Warburg apparatus at 30° in air during the period of linear oxygen uptake. Each flask contained cullular material suspended in C medium 1.0 ml., 0.1 *M* substrate 0.2 ml., carbowax in a final concentration of 10 per cent, and 20 per cent KOH 0.2 ml. in center well, total volume 2.2 ml.

Nitrogen content was determined by the micro-Kjeldahl method. The substrates used were commercial samples of analytical grade. Polyethylene glycol (Carbowax 4000) was a product of the Union Carbide and Carbon Corporation. Lysozyme was crystallized from egg white by the isoelectric point crystallization in this laboratory. Deoxyribonuclease was obtained from the Worthington Biochemical Corporation.

RESULTS

Oxidative Activities of Protoplasts, Ghosts and Supernatant—Protoplasts, ghosts and supernatant were prepared as described above. Succinoxidase, phenylenediamine (PPDA) oxidase, and oxidative activities on several tricarboxylic acid cycle members were examined with each fraction.

TABLE I

Oxidative Activities of Protoplasts, Ghosts and Supernatant

Substrate	QO_2 (N)*		
	Protoplasts	Ghosts	Supernatant
Succinate	178	105	9
Malate	19	18	7
Pyruvate	144	323	0
Citrate	33	0	9
α-Ketoglutarate	98	217	5
p-Phenylenediamine	16	49	0

* Oxygen uptake μl. per 1 mg. nitrogen per hour.

Each flask contained 20μM substrate and about 1 mg. nitrogen of cellular material.

Results are shown in Table I. With the exception of citrate almost exclusive activities of the protoplasts on these substrates were recovered in the ghosts, for instance, usually 50 per cent or more of the total activities of protoplasts recovered in the ghost fractions in the case of succinate and *p*-phenylendiamine, while in the supernatant fraction no activity was observed in these cases. When compared in the specific activity, much higher oxygen uptake was observed in the ghosts than in the protoplasts. This situation shows a concentrated localization of the oxidative enzymes to the ghosts but not to the supernatant The lock of oxidative activity on citrate of the ghosts is reasonable, because the nonsedimentable nature of aconitase and isocitric dehydrogenase is well known in many bacterial cell preparations. In preliminary experiments, it has been found that the oxidative activity of ghosts is remarkably decreased or completely lost when ghosts are suspended in a hypotonic medium, so 10 per cent carbowax was always contained in the suspending medium for the ghosts to maintain hypertonicity. But, the total activities of the protoplasts could not recovered completely in the ghosts. It may be due to the instability of the ghosts or to the inadequate assay method based on oxygen uptake.

The results indicate that in bacterial cytoplasm after cytoplasmic membrane has been removed off, there remaine no active constituents concerning on the terminal oxidation. Further experiments to confirm the assumption, that the active particles obtained after mechanical disruption of the bacterial cells might be derived from the cytoplasmic membrane and not exist *in situ* in the cytoplasm, were designed.

Table II

Activities of Subcellular Particles Derived from Intact Cells and Ghosts by Grinding with Glass Powder

Substrate	QO_2 (N)				
	Crude extract	Ghosts	P_1	P_2	S_2
Succinate	23		17	67	0
,,		67	18	64	10
p-Phenylenediamine	40		107	403	0
,,		25	45	55	0

Each flask contained 20 μM subrtrate and about 0.5 mg. nitrogen of cellular material.

Particles from Intact Cells and Ghosts—Intact cells and ghosts were similarly treated to disrupt either by grinding with glass powder or by sonic oscillation. After preparative ultracentrifugation, both disrupted cellular extracts were separated into S_1 and P_1 fractions, and then to S_2 and P_2 fractions respectively.

As already has been presented by Weibull (*2*), cytochromes were localized to the ghosts and recovered in P_1 and P_2 fractions but not in S_2 fraction in both cases when different sources were employed for mechanical disruption. Localization of succinoxidase and PPDA oxidase were examined with these subcellular fractions derived from either intact cells or ghosts. The average results of several experiments are shown in Tables II. and III. When disruption was performed by grinding with glass powder, as shown in Table II, succinoxidase and PPDA oxdase are localized completely to the P_1 and P_2 fractions, and not detected in the S_2 fraction in both case except a negligible activity is distributed in the S_2 fraction derived from the ghosts, From these results the proposal that the active particles are originated from the ghost is significantly certified.

TABLE III

Activities of Subcellular Particles Derived from Intact Cells and Ghosts by Sonic Oscillation

Substrate	QO$_2$ (N)				
	Crude extract	Ghosts	P_1	P_2	S_2
Succinate	30		12	68	10
,,		37	83	37	8
p-Phenylenediamine	20		39	77	4
,,		25	34	50	32

Each flask contained 20 μM substrate and about 0.5 mg. nitrogen of cellular material.

But in the case when sonic oscillation was employed as a disrupting method, the localization of succinoxidase together with PPDA oxidase is not clear. As shown in Table III, considerable parts of activities were distributed to the S_2 fractions in both cases. The difference brought by two different disruption methods may be explained by the more thorough disruption effect of the sonic oscillation. Sonic treatment might disintegrate the intact cells or the ghosts into more fine particles which were no longer sedimentable by the centrifugal forces employed. This is just in accord with the result obtained by Repaske (5) on the succinic dehydrogenase of *Azotobacter vinelandii.*

DISCUSSION

In the previous report (*6*), concerning to the development of *Bacillus cereus*, and observation was described in the particulate fraction which contained almost exclusive cytochrome components and terminal oxidative activities of the whole cells. The organelle nature of the particle-bound respiratory enzyme system was gradually established concomitantly to the development of the cells, namely, development from the germinated stage the vegetative growth stage.

The purpose of this work is to confirm the site or the constituent from which these active particles are derived. Already Stanier (*7*) has proposed his thought concerning to the bacterial particles based on the experimental results reported by Weibull (*1*, *2*), that "the bacterial cytochrome system, along with some other enzymes, may be abuilt-in part of the cell membrane, and not, as in higher organisms, a mitochondrial component". Weibull's findings provide an information that the sonic-treated ghost material is similar in its physical properties to the particulate fractions which have been obtained by mechanical disruption of the many bacteria. The similarity of the enzymatic activity between the disintegrated ghost material and the particulate fraction of the intact cells has been pointed out in the present work. A concentrated localization of terminal oxidase system to to ghosts has been also presented.

Thus, in accords with Stanier's proposal, the active particles may be a buit-in part of the cytoplasmic membrane and not exist in the cytoplasm as discrete constituents.

SUMMARY

In the cells of *Bacillus megaterium* KM, succinoxidase, *p*-phenylenediamine (PPDA) oxidase and oxidative activities on the several members of the tricarboxylic acid cycle are localized to to the ghosts.

The similarity of the active particles derived from either intact cells or ghosts was confirmed on succinoxidase and PPDA oxidase.

Relationship between the ghosts and the active particles was discussed.

REFERENCES

(*1*) Weibull, C., *J. Bacteriol.*, **66**, 688 (1953)
(*2*) Weibull, C., *J. Bacteriol.*, **66**, 696 (1953)
(*3*) Weibull, C., In Bacterial Anatomy, 6th Symposium of the Society for General Microbiology, University Press, Cambridge, England, 111, (1956)
(*4*) McQuillen, K., *Biochim. Biophys. Acta.* **17**, 382 (1955)
(*5*) Repaske, R., *J. Bacteriol.*, **68**, 555 (1954)
(*6*) Nakada, D., Matsushiro, A., Kondo, M., Suga, K. and Konishi, K., *Med. J. Osaka Univ.*, **7**, 809 (1957)
(*7*) Stanier, R.Y., *I*n Cellular Metabolism and Infection, Academic Press Inc., New York 3 (1954)

ADDENDUM

The present work has been already announced at the Annual Meeting of the Japanese Biochemical Society on July 15th 1957 in Kyoto. Before the contribution of this work a, similar articles by Storck and Wachsman (Storck, R. and Wachsman, J.T., *J. Bacteriol.*, **73**, 784 (1957)) has appeared, which is in agreement with the present work.

The Journal of Biochemistry, Vol. 45, No. 10, 1958

METABOLISM OF PARA-AMINOSALICYLIC ACID

IV. STUDIES ON THE METABOLISM OF META-AMINOPHENOL IN REFERENCE TO THAT OF PARA-AMINOSALICYLIC ACID

By MAKOTO NAKAO, ISAMU YANAGISAWA AND
HARUHISA YOSHIKAWA

*(From the Department o Physiological Chemistry and Nutrition,
Faculty of Medicine, University of Tokyo, Tokyo)*

(Received for publication, November, 7, 1957)

The metabolic products of *p*-aminosalicylic acid in the urines of human beings and other animals were described in the preceding papers. (*1–3*) There remains a possibility that the degradation of *p*-aminosalicylic acid (PAS) in animal body may take place through *m*-aminophenol, since PAS is readily decarboxylated *in vitro* upon heating with acid. Venkataraman *et al.* (*4*) and Tsukamoto and Yamamoto (*5*) concluded formerly that there was no indication of decarboxylation of PAS in rabbit since *m*-aminophenol could not be detected in the urine of the animals to which PAS has been administered. However, this conclusion seems to be premature, because, as demonstrated by Williams (*6*) and Bray *et. al.* (*7*) *m*-aminophenol which is given to animal is excreted totally conjugated forms and therefore, non existence of free *m*-aminophenol in the urine of the animal dosed with PAS might be attributed to that *m*-aminophenol is converted to the detoxication products as soon as formed from PAS in the animal body, as suggested by Kawamata Kashiwagi (*8*). The authors describe in this paper the metabolic products of *m*-aminophenol, and report that none of them can be detected in the urine after admininistration of PAS.

MATERIALS AND METHODS

A half gram of *m*-aminophenol or PAS per kg. body weight was administered to a rabbit through a stomach tube or to a mouse by intraperitoneal injection. The urines excreted thereafter for 24 hours were collected and treated in the same way as described in the previous paper (*1*) for paper chromatography. Two-dimensional paper chromatograms were run first *n*-butanol-acetic acid-water (4 : 1 : 5) and then in methanol-butanol-benzene- water (2 : 1 : 1 : 1), and after drying, sprayed with Ehrlich's reagent or Bratton Marshall's diazo reagents to locate the spots and to elucidate whether the aromatic amino group was free or not.

In order to detect the sulfate conjugate of *m*-aminophenol, about 50 μc of S^{35}-sulfate was given to a mouse together with 5 mg. of *m*-aminophenol by intraperitoneal injection and the urine was collected for 24 hours. After the urine was desalted by the addition of 95 per cent ethanol and centrifugation, the supernatant was concentrated in vacus to the original volume and subjeted to two-dimensional paper chromatography as described above. The dried paper was sprayed with Ehrlich's reagent, dried again and kept in contact with Fuji No-Screen Film in a cassettee for about two weeks

RESULTS AND DISCUSSION

As a preliminary experiment in vitro decarboxylation of PAS was studied by heating its solution in 0.1 *N* HCl at 100°. The decomposition was followed up spectrophotometrically, the optical densities being measured at 300 mμ and 265 mμ, the wave lengths of maximal absorption of PAS and *m*-aminophenol respectively (Fig. 1). It was shown that more than 90 per cent of PAS was converted to *m*-aminophenol within 20 minutes. This fact indicates that a great care must be taken to avoid the decarboxylation of unchanged PAS as well as of PAS derivatives during the treatment of the urine containing the metabolic products of PAS.

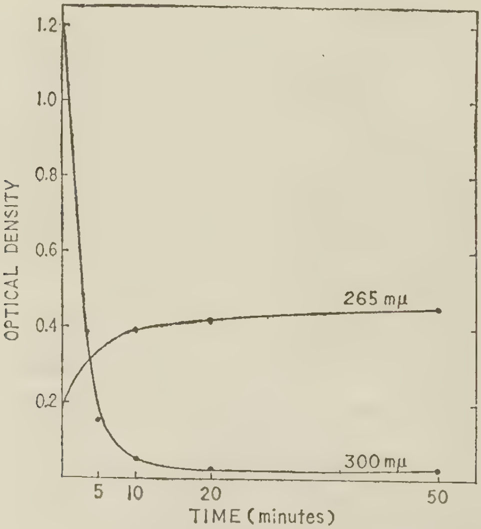

Fig. 1. Time course of decarboxylation of PAS by heating in 0.1 *N* HCl at 100°.

If the decarboxylation of PAS took place to any extent in the animal body, the urine excreted after administration of PAS would contain substances derived from *m*-aminophenol. When *m*-aminophenol was administered to mice and the collected urine was analyzed by paper chromatography, six spots due to *m*-aminophenol derivatives were seen on the paper, as shown in Fig. 2. These spots were numbered for convenience in the order of R_f values on the chromatogram developed with methanol-benzene-*n*-butanolwater. Besides these spots there appeared an unspecified faint blue spot after spraying with Bratton Marshall's reagents and a diffuse spot of faint yellow color due to urea with Ehrlich's reagent, as described before (*1*).

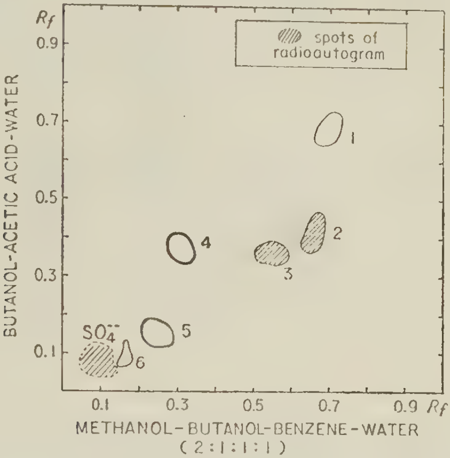

FIG. 2. Two dimensional chromatogram of metabolic products in mouse urine.

The R_f values of the spot No. 1 agreed well with those of an authetic sample of *N*-acetyaminophenol. Although the position of the spot is near that of *m*-aminophenol, this spot was stained yellow several minutes after spraying with Ehrlich's reagent, while the spot of an authenic sample of *m*-aminophenol was coloured promptly. This spot No. 1 did not react with Bratton Marshall's diazo reagents. From these findings it is reasonably concluded that this spot was of *N*-acetylaminophenol and that free *m*-aminophenol did not exist in the urine. The spots No. 2 and 3 with R_f values of 0.40 and 0.35 in butanol-acetic acid-water coincided in their position and shape with the radioactive spots obtained from the urine of a mouse given S^{35}-sulfate and *m*-aminophenol. The spots No. 3 was stained immediately by spraying with Ehrlich's reagent and with Bratton Marshall's reagent, while the spot No. 2 appeared a few minutes later with Ehrlich's regent. These facts suggest that the spot No. 2 is due to *N*-acetylaminophenyl sulfate and the spot No. 3 to *m*-aminophenyl sulfate.

The spot No. 5 gave a prompt positive Bratton Marshall's reaction, as well as Ehrlich's reaction but the spot No. 4 gave positive reaction with Ehrlich's reagent a few days after spraying the reagent and no coloration with Bratton Marshall's reagents. The Eluates

TABLE I

Metabolic Products of m-Aminophenol in Mouse Urine

Spot No.	R_f value		Color reaction		After 1 N H_2SO_4 hydrolysis at 100° for 15 minutes	Name of compound
	Butanol-acetic acid-water 4:1:5	Methanol-butanol-benzene-water 2:1:1:1	Bratton Marshall's reagents	Ehrlich's reagent		
1	0.70	0.70	—	Yellow (delayed)	Disappeared	*N*-acetyl-aminophenol
2	0.40	0.65	—	Yellow (delayed)	Disappeared	*N*-acetyl-aminophenyl-sulfate
3	0.35	0.55	Dark purple	Yellow	Disappeared	Aminophenyl sulfate
4	0.40	0.30	—	Yellow (delayed)	Disappeared	*N*-acetyl-aminophenyl glucuronide
5	0.15	0.28	Dark purple	Yellow	Unchanged	Aminophenyl glucuronide
6	0.05	0.18	—	Yellow	Disappeared	Unknown
*	0.68	0.68	Dark purple	Yellow	Appeared	Unchanged aminophenol

* An authentic sample.

TABLE II

Metabolic Products of m-Aminophenol in the Urine of Various Animals

	Name of compound	Cog	Rabbit	Mouse
1	*N*-acetylaminophenol	—	+	+
2	*N*-acetylaminophenyl sulfate	—	+	+
3	Aminophenyl sulfate	+	+	+
4	*N*-acetylaminophenyl glucuronide	—	+	+
5	Aminophenyl glucuronide	+	+	+
6	Unidentified	+	+	+
	Unchanged aminophenol	—	—	—

of the two spots, No. 4 and gave violet color by treating with naphtoresorcinol. Moreover, the spots Nos. 1, 2, 3. and 4 disappeared by acid hydrolysis, whereas the spot No. 5 remained unchanged. These results suggest that No. 4 is the spot of *m-N*-acetylaminophenyl glucuronide and No. 5 is that of *m*-aminophenyl glucuronide.

The character of No. 6 could not be examined further, as this spot was so faint and small. These results are summarized in the Table I.

Since metabolic pattern of detoxication of PAS was different among various animals as reported before (*3*), the metabolic fate of *m*-aminophenol was studied in rabbit, mouse and dog, as shown in Table II.

The urine samples of rabbits gave almost the same spots as those of mice, but the dog did not give the spots Nos. 1, 2 and 4, in accordance with the general rule that dog does not excrete aromatic amines in *N*-acetylated form.

Non existence of the metabolic products of *m*-aminophenol in the urine of animals to which PAS was administered.—If the decarboxylation of PAS occurred in the animal body in any extent, the metabolic products of *m*-aminophenol would be demonstrated in the urine after administration of PAS to the animal. This was not the case. The metabolic products of PAS identified in human urine after its oral administration (*1*, *2*) are summed up again in the Table III. The

TABLE III

Characteristics of Each Metabolic Products on the Chromatogram of Human Urine after Oral Administration of PAS

Supposed name of compound	R_f value		Bratton-Marshall's reaction	After acid hydrolysis	Radioautogram of the chromatogram of the urine after administration of $S^{35}O_4$ and PAS
	In butanol-acetic acid water (4 : 1 : 5)	In methanol-benzen-butanol-water (2 : 1 : 1 : 1)			
N-acetyl PAS	0. 28	0. 60	—	Disappeared	–
PAS	0. 79	0. 50	R	Appeared	–
p-Aminosalicyluric acid	0. 46	0. 38	R-V	Appeared	–
Ester glcuronide of *N*-acetyl PAS	0. 38	0. 55	—	Disappeared	–
Ether glucuronide of N-acetyl PAS	0. 33	0. 35	—	Disappeared	–
Ester glucuronide of PAS	0. 28	0. 35	R	Appeared	–
Sulfate conjugate	0. 28	0. 32	BI-V	Disappeared	+
Unknown glucuronide	0. 19	0. 22	R	Disappeared	–
Ether glucuranide of PAS	0. 14	0. 12	R-V	Appeared	–

In this table, the following abbreviations are used: blue (Bl), red (R), violet (V).

comparison of the metabolic products of PAS and *m*-aminophenol revealed that there was no compound common to them, as in the following way.

A) PAS gave only one spot of sulfate conjugate, whereas *m*-aminophenol two.

B) The R_f valves of the sulfate conjugate of PAS were different from either of those of the sulfate conjugates of *m*-aminophenol and its *N*-acetyl derivative as shown in Table I. By spraying with Bratton Marshall's reagent the spot of sulfate conjugate derived from PAS developed a blue violet to blue color, while the spots from *m*-aminophenol developed a dark purple colcor.

C) The glucuronides derived from PAS possesed R_f values which were quite different from those of the glucuronides of *m*-aminophenol and its derivative in the urine. The color of the formers was red violet, while that of the latters was dirty purple.

D) In the urine of mouse to which PAS was administered, glucuronides of PAS and *N*-acetyl PAS and unidentified glucuronide were found, but no sulfate conjugate, whereas two glucuronides as well as two sulfate conjugates were excreted in the urines of rabbit and mouse dosed with *m*-aminophenol.

SUMMARY

Metabolism of *m*-aminophenol in mouse, dog and rabbit were examined by paper chromatography. *N*-acetylaminophenyl glucuronide, aminophenyl glucuronide, N-acetylaminophenyl sulfate, aminophenyl sulfate and an unidentified compound were excreted in the urine of mouse and rabbit. *m*-Aminophenyl glucuronide and *m*-aminophenyl sulfate were excreted in the urine of dogs given *m*-aminophenol. But unchanged *m*-aminophenol was not excreted in any case. The metabolic products of *m*-aminophenol were not recognized in the urine of animals to which PAS was administered, so far as the excreted urine was analysed by means of paper chromatography. These results led us to the conclusion that PAS is not metabolized through *m*-aminophenol in appreciable amounts.

REFERENCES

(*1*) Nakao, M., *J. Biochem.*, **4**, 327 (1957)
(*2*) Nakao, M., and Yanagisawa, I., *J. Biochem.*, **44**, 433 (1957)
(*3*) Nakao, M., Yanagisawa, I. and Yoshikawa, H., *J. Biochem.*, **44**, 477 (1957)
(*4*) Venkataraman, A., Venkataraman, P.R., and Lewis, H.B., *J. Biol. Chem.*, **173**, 641 (1948)
(*5*) Kawamata, J. and Kashiwagi, K., *Med. J. Osaka Univ.* **6**, 111 (1955)
(*6*) Williams, R.T., *Biochem. J.*, **28**, 878 (1938)
(*7*) Bray, H.G., Clowes, R.C., and Thorpe, W.V., *Biochem. J.*, **51**, 70 (1952)
(8) Tsukamoto, H., and Yamamoto, A., *Pharm. Bull.* (*Japan*), **3**, 427 (1955)

The Journal of Biochemistry, Vol. 45, No. 10, 1958

SOME OBSERVATIONS ON GLYCEROL-TREATED MUSCLE FIBERS*

By SHIZUO WATANABE** AND WILLIAM SLEATOR, JR.

(*From the Department of Physeology, Washington University School of Medicine, St. Louis, Mo.*)

(Received for publication, April 14, 1958)

The effect of magnesium, calcium, and managanese on the relaxation of glycerol-treated rabbit psoas muscle fibers produced by ethylene diamine tetraacetic acid (EDTA)*** (*1*, *2*) and by isopropylene diamine tetraacetic acid (PDTA) have been reported in a previous paper (*3*). Among the results of this work was the finding that, altnough Mn^{++} can substitute for Mg^{++} in ATP induced contractions. neither Ca^{++} nor Mn^{++} can take the place of Mg^{++} in EDTA (or PDTA) relaxations. Weber (*4*) has found that inorganic pyro- and tri-phosphate also have a relaxing effect on glycerol-treated fibers, and Bendall (*5*) and Bozler (*1*) reported that this relaxation has a divalent ion requirement which can be satisfied by Mg^{++}. One of the purposes of the present work was to investigate the specificity of this "Mg^{++} requirement" in pyrophosphate relaxation as was previously done for EDTA relaxation.

Another purpose of the present work was to study the effect of cysteine on glycerinated fibers (it also produces a lengthening which is accelerated by Mg^{++}), and the influence of various divalent ions and of monoiodoacetate on this cysteine effect.

A series of experiments will also be reported in which attempts were made to establish a preparation procedure which would consistently produce fibers showing incomplete relaxation by EDTA. In addition, various attempts were made to restore completeness of EDTA relaxation by use of cysteine, and by altering such variables as pH and amount of shortening. These experiments are of some interest in connection with the second relaxation factor reported by Kumagai, Ebashi and

---

* This investigation was supported by grant G 2657 from the Division of Research Grants of the National Institutes of Health, U.S.P.H.S. (1956)

** Present address: Department of Chemistry, Hokkaido University Faculty of Science, Sapporo, Japan.

*** In this paper, the following abbreviations are used: ATP, Adenosinetriphosphate; ATPase, Adenosidetriphosphatase; EDTA, Ethylenediamine tetraacetic acid; PDTA, iso-Propylenediamime tetraacetic acic; TMP, 2,4,6-Trimethylpyridine; IAA, Mono-iodoacetic acid; DNP, 2,4-Dinitrophenol.

Takeda (*6*). Also described are the effects (or lack of them) of several enzyme inhibitors, and of acetyl-choline and histamine; and finally, the results of a few experiments with fibers from an invertebrate smooth muscle.

METHODS

With the two exceptions noted later, the experiments reported here were carried out with glycerol-treated fiber bundles from rabbit psoas muscle. The preparation procedure to which we refer here after as "standard" was the same as that used in the previous paper (3) and many be outlined as follows:

Fiber bundles of fresh rabbit psoas muscle about 3 mm. in diameter were treated with a 50 per cent glycerol-water solution at 0° for 2 days and then at about −15° for 50 to 90 days. Then fiber bundles about 0.4 mm. in diameter were separated from the larger bundles, and washed in 260 mM per liter KCl at 0° for several hours. Each Experiment was done with one of the small bundles in a 10 ml. bath stirred gently by bubbling nitrogen, at a room temperature of about 26°. Changes in length of the fibers were recorded on a kymograph with an isotonic lever loaded to about 150 g. per cm^2 cross section and compared to the initial length of the fiber under this load.

The cysteine used was obtained from the Fisher Scientific Co. in the mono-hydrochloric form, and neutralized with KOH just before use. The mono-iodoacetic acid was prepared by recrystallizing the Eastman product twice, ethyl using ether and petroleum ether; it also was neutralized with KOH for use. Trimethyl pyridine (TMP) at aconcentration of 50 mM per liter was used as a buffer in all experiments except part of those described in section 5.

The "standard contraction medium" consisted of 150 mM KCl containing 4 mM ATP, 4 mM $MgCl_2$ and buffered with 50 mM TMP (pH 6.95) per liter. The standard relaxation medium contained 4 mM ATP, 4 mM $MgCl_2$, 4mM EDTA in 140 mM KCl buffered with TMP (pH 7.05) per liter.

RESULTS

1. Effects of Ca^{++} and Mn^{++} on Relaxation by Pyrophosphate—The first experiments to be considered here deal with whether either calcium or manganese can take the place of magnesium in enabling pyrophosphate to produce relaxation of standard glycerol-treated fibers. As shown in Fig. 1, curves a and b*, when the standard contraction medium is replaced by one consisting of 20 mM sodium pyrophosphate in 150 mM KCl buffered with TMP, pH 6.95, per liter, the contracted fiber lengthens a small amount. According to Bozler (*1*), this slight lengthening is attributable to Mg^{++} introduced during the contraction, and remaining on the fiber for awhile in the new medium. In Curve a of Fig. 1, 4 mM per liter Mn^{++} was then added to the pyrophosphate medium with no effect, while subsequent addition of 4 mM per liter Mg^{++} to the medium caused an accelerated relaxation of the fiber. Similarly, Ca^{++} is unable to play the role of Mg^{++} in pyrophosphate relaxation, as demonstrated in Curve b of Fig. 1. However, Ca^{++} often does have some sort of damaging effect on the fiber; the dotted line following Curve b represents the irreversible lengthening of the fiber which would probably have occurred if Mg^{++} had not been added.

---

* Here, as in all of the figures of this paper, a +sign before a symbol along a curve means that the substance symbolized was added to the fiber medium at that time. Where symbols not preceded by +signs appear, it means that a new medium containing the substances indicated (in 150 mM per liter KCl with TMP buffer, pH 6.95) was exchanged for the previous medium. "Wash" means that the fiber was immersed in 160 mM per liter KCl aq. The washings lasted until the next solution was applied, so their durations can be read on the time scale.

The accelerating effect of ATP on the pyrophosphate relaxation, which has been reported by Bendall (*5*), and by Bozler (*1*) for his "fresh fiber" preparations, is not observed with fibers prepared in our standard manner. On the contrary, in our experiments, as in those reported by Weber (*4*), relaxation by pyro- or triphosphate is generally reversed by addition of ATP (Fig. 1, end of Curve c)

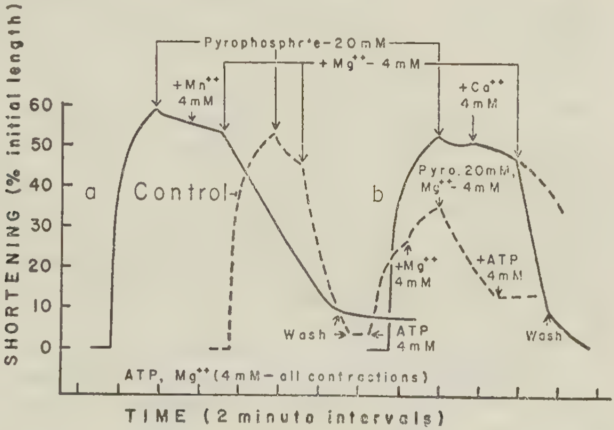

FIG. 1. The effect of Mg^{++}, Ca^{++} Mn^{++} ions on pyrophosphate relaxation. The "control" curve (dotted) shows the effect of pyrophosphate with Mg^{++} alone, as fiber is taken through 2 cycles of contraction and relaxation. Curves a and b show that neither Mn^{++} nor Ca^{++} can substitute for Mg^{++} in the relaxation process.

Three points of eifference between pyrophosphate relaxation and that induced by EDTA (with ATP) should be noted here: (1) Washing with 160 mM per liter KCl, after relaxation in an inorganic polyphosphate medium without ATP, does not cause any shortening of the fiber such as occursaf ter FDTA relaxations (see Fig. 6 for examples). (2) Neither Ca^{++} nor Mn^{++} has a reversing effect on the pyrophosphate relaxation, such as they do on EDTA relaxation. (These two difference are probably due to the fact that ATP is always present in the EDTA case and is not supplied in the pyrophosphate case.) (3) After inorganic polyphosphate relaxation and washing in 160 mM per liter KCl, a second shortening by ATP+Mg^{++} is always substantially smaller than the first one (Fig. 1, Curve c, second contrn.)

2. *Effects of Cystein*—Under certain "uncontrolled" conditions glycerol-treated fibers show this sort of abnormal behavior; they contract, but only partially, in the KCl medium with ATP and Mg^{++}, and they lengthen spontaneously following such partial contraction. If this lengthening were due to the continued presence of some "natural" relaxation factor, for example myokinase, it should be reversed completely by addition of Ca^{++}, and partially by washing with 160 mM per liter KCl aq. However, such lengthening is actually accelerated by Ca^{++} addition, and is not completely stopped by washing in KCl. (For these reasons we will use the word lengthening rather than relaxation to refer to this process.)

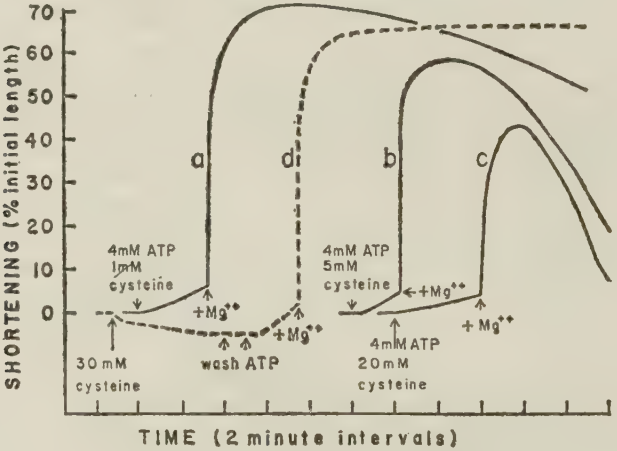

Fig. 2. Lengthening effect of cysteine. After glycerol treatment these fibers were washed at 0° in 160 mM per liter KCl aq. for 20 to 30 hours before use. In Curves a, b and c, cysteine was applied at concentrations of 1 mM, 5 mM, and 20 mM per liter the contraction (4 mM ATP and 4 mM Mg^{++} per liter); the fibers subsequently relaxed at increasing speeds without any change in the medium. The fiber giving Curve d was washed for 1 minute after exposure to 30 mM per liter cysteine, and showed no tendency to lengthen after its normal contraction.

This kind of abnormal behavior can be produced in normal fibers (prepared in the standard way) by addition of cysteine to the medium. As shown in Fig. 2, curve a, cysteine can do this at concentrations as low as 1 mM per liter, though its effectiveness also depends on the thickness of the fiber. Curves b and c of Fig. 2 show the earlier starting and faster lengthening which follow contractions when 5 mM or 20 mM per liter cysteine has been applied to the fibers along with the ATP. In Curve d, a fiber exposed to 30 mM per liter cysteine for 10 minutes was washed briefly before contraction by ATP and Mg^{++}. It fails to show any spontaneous relaxation, suggesting that the cysteine effect may not be its reduction effect of un contracted fibers.

The effect of cysteine on these fibers studied further in the experiments shown in Fig. 3. Here Curve a shows that cysteine alone in KCl can produce lengthening of a fiber shortened by ATP and Mg^{++}.

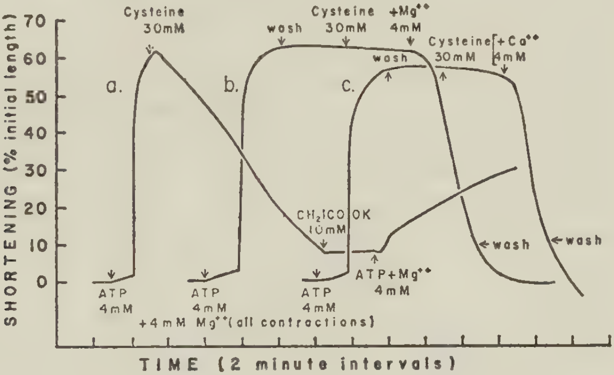

FIG. 3. Eeffect of Mg^{++} and Ca^{++} ions on the cystene lengthening. In curve a 30 mM per liter cysteine alone produced lengthening; when the fiber was washed before cysteine application, as in curves b and c, lengthening was insignificant, but could be produced by addition of Mg^{++} or Ca^{++}.

Cysteine is much less effective after the contracted fiber has been washed in 160 mM per liter KCl (Curve b) but the addition of Mg^{++} (4 mM per liter) accelerates the lengthening markedly. Therefore, it is probable that the lengthening which occurs when cysteine alone is added (as in Curve a) depends on Mg^{++} introduced during the contraction and remaining on the fiber after the media have been exchanged. In contrast to relaxation by EDTA, pyrophosphate, and the others factors, lengthening by cysteine is not specific in its divalent ion requirement. Cerve c of Fig. 3 shows that Ca^{++} is just as effective as Mg^{++} in accelerating the cysteine lengthening.

3. *Effect of Monoiodoacetate* (*IAA*)—The lengthening of glyrcerol-treated fibers by cysteine is stopped only slowly or not at all by washing in 160 mM per liter KCl aq., but it is stopped immediately by the addition of IAA (Fig. 3, Curve a). Also, fibers lengthened by cysteine often show no shortening or only a slight amount on subsequent application of ATP and Mg^{++}. However, when a cysteine lengthened fiber is treated with

IAA and then exposed to a medium containing ATP with or without Mg^{++}, or when IAA and ATP are applied together, a considerable amount of slow shortening is always observed (Fig. 3, end of Curve a).

In the experiment shown in Fig. 4, Curve a, 10 mM per liter potassium monoiodoacetate was added to the standard contraction medium before it was applied to the fiber. The relaxation was carried on in the standard EDTA relaxation medium, and was only about 30 per liter of normal in this typical case, thought the contraction was normal or slightly accelerated (compared with dotted control curve). In Curve b (Fig. 4) 10 mM per liter IAA was added to the relaxation medium instead of to the contraction medium. In this case, relaxation is scarcely influenced by the added ion. Curve c shows the effect of exposing the fiber to IAA for 3 minutes and washing in KCl for 1 minute before applying the standard contraction medium; the application and removal of IAA before shortening show some but much less effect on relaxation than application during shortening. The attempt has been made to restore, or produce, complete relaxation of fibers contracted in iodoacetate (as in Curve a) by application of cysteine, but it has not been successful so far.

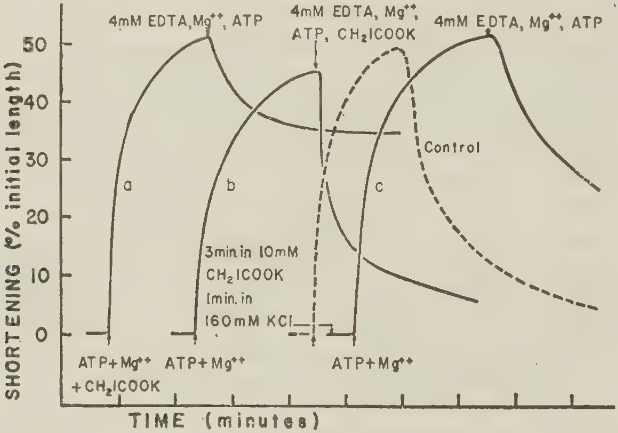

Fig. 4. Effect of mono-iodoacetate on contraction and relaxation. Curve a: 10 mM per liter IAA added to standard contraction medium (ATP and Mg^{++}, 4mM per liter). Curve b: 10 mM per liter IAA added to standard relaxation medium. Curve c: fiber exposed to 10 mM per liter IAA and then washed far 1 min. before contraction. Control curve (dotted): no IAA.

4. *Observations on Incomplete EDTA Relaxation*—There have been some glycerol-treated fibers prepared by our standard procedure which contract prefectly (rapidly and up to 70 per cent of initial length under the normal load of 150 to 200 g./cm^2) but which relax only incompletely in

the standard EDTA relaxation medium. Since Kumagai *et al.* (*6*) have reported that a "second factor" is involved in relaxation of glycerinated fibers, this incompleteness of EDTA relaxation could be due to lack of the second factor in some of oui fiber preparations. In order to study this question further, fibers which consistently lacked the second factor were needed, and their preparation was undertaken by several methods.

(a) The glycerol-treatment was carried out with muscle bundles about 2 mm. in diameter (instead of the standard 3 mm); they were kept in 50 per cent glycerol-water at 0° for 40 hours (standard is 20 hours) with two renewals of the solution instead of one; and after transfer to the deepfreeze at −10° the solution was renewed once a week (instead of not at all).

After a number of days (which was one of the variables to be studied) these fiber bundles from the deep freeze were divided into smaller ones about 0.2 mm. in diameter, and put into 160 mM per liter KCl solution at 0°. Half an hour later the KCl solution was renewed and the washing at 0° continued for a number of hours (which was the other experimental variables). The fibers were then put briefly into 160 mM per liter KCl at room temperature (about 26°) before being exposed to the standard contraction and relaxation madia (contraction: 4 mM ATP and Mg, 50 mM buffer per TMP liter, relaxation: some plus 4 mM EDTA per liter.)

A group of fibers which was stored at −10° for 12 days and washed at 0° for several hours already show a behaviour similar to that of fibers prepared in the standard way (which involves storing at −10° for 30 to 60 days). The curves of Fig. 5 show the behavior of fibers stored at −10° for 58 days, and except for the first (which was washed for only 0.5 hours) they all show marked incompleteness of EDTA relaxation. The curves of Fig. 5 are examples from a series of experiments which lead to the following generalization:

(1) When the storage period at −10° is less than about 30 days the fiber is "fresh" and the duration of the 0° washing in KCl makes little difference in behavior (EDTA relaxation is always complete) while it improves the contraction.

(2) Even with fibers which have been stored at −10° for 2 months ("old" fibers) about 10 hour washing in 160 mM per liter KCl at 0° is necessary to see consistetly the incomplete EDTA relaxation; beyond this, the longer the washing, the greater the incompleteness.

(3) Continuing the 0° 160 mM per liter KCl washing for more than 48 hours usually caused a decrease in the amount of contraction, as seen in the last curve of Fig. 5, but these fibers still show some EDTA relaxation. In short, we were unable to find any combination of storage and washing durations which would lead to complete contraction but negligible relaxation by the EDTA medium.

(b) Another set of experiments was carried out in the following way;

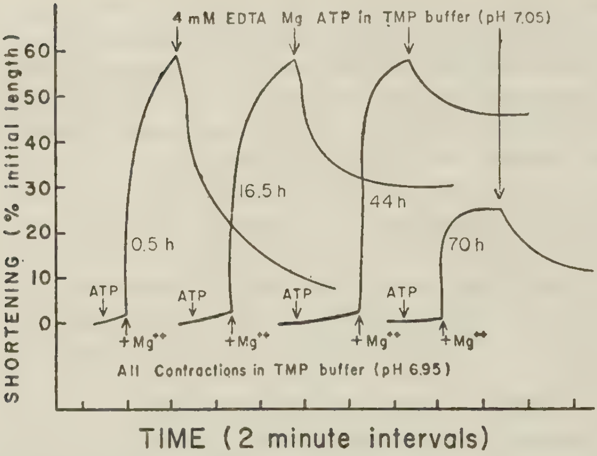

FIG. 5. Effect of washing time in 160 mM per liter KCl aq. on contraction and relaxation of old fibers. All fibers used here had been kept in 50 per cent glycerol-water at —10° for 58 days. Curve a: This fiber was washed in 160 mM per liter KCl aq. for 0.5 hours; Curve b: 16.5 hours washing; Curve c: 44 hours; and Curve d: 70 hours

after glycerol-treatment the fibers were first washed in 150 mM per liter KCl containing 10 mM per liter K_3EDTA for hours, and then given the 0° washing in 16 0mM per liter KCl aq. for about 60 hours. These fibers were found to contract completely in the standard contraction medium, and to show only a little incompleteness of EDTA relaxation. The fact that EDTA treatment protects fibers from the usual effects of long washing (as shown in Fig. 5, last curve) suggests that the presence of bivalent cations (probably Mg^{++}) on the fiber tends to accelerate whatever process goes on during washing.

(c) During previous experiments (*3*) it was observed that when contraction and relaxation are repeated at pH 8 (TMP buffer) the second or the third relaxation is more likely to show incompleteness than when the processes are carried out at pH 6.3 (*2*). It has also been found that when a fiber is allowed to shorten completely (60–70 per cent of initial length) and to remain short for 10 minutes or more in the standard contraction medium (ATP and Mg^{++} at pH 7.0) in most cases its subsequent relaxation in the EDTA medium is incomplete. Therefore it might be expected that carrying out the 0° washing of the fibers in 160 mM per liter KCl buffered with 50 mM per liter TMP at pH 7 would lead to more incompleteness of EDTA relaxation than occurs after a long washing in 160 mM per liter KCl aq., pH about 6.2*. The experiments were undertaken to test this possibility. Incompleteness of EDTA relaxation appears to increase with washing time just about as it does for "standard" fibers, while contraction is usually somewhat slower than standard. Here also, as the duration of the 0° washing is increased, the size of the contraction decreases before the capacity for EDTA relaxation disappears completely.

---

* Along these lines, we might also have considered the possibility that ATP or ATP+Mg^{++} makes the extraction of the second factor more complete, but at present we have no data on this question.

According to a suggestion by M.C. Goodall (unpublished communication) psoas fibers prepared with ethylene glycol instead of with glycerol may respond differently to the EDTA relaxation medium. Therefore we prepared some fibers by a procedure which was standard except that the 50 per cent glycerol was replaced by a 50 per cent ethylene glycolwater solution. The fibers were kept in this solution at −10° for about 30 daysand prepared in the usual way for experiments which showed: (1) These fibers contract very nicely in the standard 4 mM per liter ATP plus Mg^{++} contraction medium and (2) They relax in the standard EDTA medium as well as glycerol trated fibers do. To summarize, our experiments did not show any significant differences between the ethylene-glycol- and the glycerol-treated fibers except that the former are markedly less transparent than the latter.

5. Attempts to Restore Completeness of EDTA Relaxation—A number of experiments were carried out with the older well-washed fibers described above (more than 50 days at −10° in glycerol and more than 10 hours 0° washing in KCl aq.) for the purpose of determining what conditions, if any, could abolish the incompleteness of EDTA relaxation which they usually show.

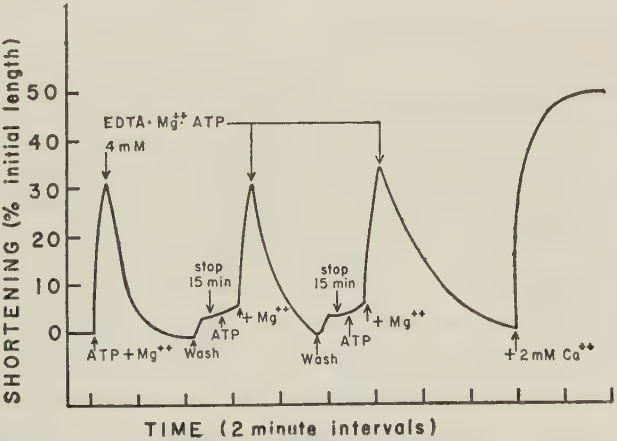

FIG. 6. Effect of early application of relaxation medium. The standard media were used for both contraction (4 mM per liter ATP and Mg^{++}) and relaxation (4 mM per liter ATP, Mg^{++}andEDTA) but the relaxation medium was applied when contraction reached 30–35 per cent of initial length.

(a) In the experiment of Fig. 6, the standard EDTA relaxation medium was applied relatively early in the contraction, when the shortening had reached about 30 per cent of initial length ("physiological range") rather than the usual 60–70 per cent. Under these conditions complete relaxation was always obtained with the EDTA medium, even after two full cycles of contraction and relaxation. (The dotted control curve in Fig. 7 shows the incomplete relaxation of these fibers which would follow a complete contraction). The only significant differences between the 3 cycles shown in Fig. 6 are that relaxation (and to some extent contraction) tends to become slower each time. After the 3rd complete relaxation the fiber contracts rapidly by 50 per cent in Ca^{++} indicating no extraction of or damage to contractile proteins.

(b) The effect of cysteine on the incomplete EDTA relaxation was studied in the experiments of Fig. 7. In Curve a, 20 mM per liter cysteine was added to the standard relaxation medium, with no significant effect (compare with dotted control curve). Since lengthening by cysteine requires Mg^{++}, an excess of Mg^{++} over EDTA might be needed to see any effect of cystein. In Curve b, 8 mM per liter Mg^{++} (instead of the standard 4 mM) was used in the relaxation medium containing 20 mM per liter cysteine. Here we always see some relaxation beyond what would have occurred without cysteine, but it is not yet complete.

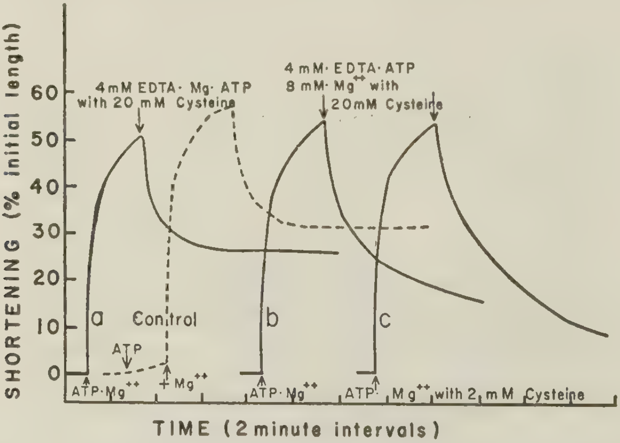

FIG. 7. Effect of cysteine on incomplete EDTA relaxation. The dotted curve is the control in standard contraction and relaxation media. For the other curves, 20 mM per liter cysteine was added to the relaxation medium, and for Curve c, 2 mM per liter cysteine was also present during the contraction.

In the experiment leading to Curve c (Fig. 7), 2 mM per liter cysteine was added to the contraction medium, and the relaxation medium, containing 20 mM per liter as befor, was applied to the fiber before the "spontaneous" cysteine lenghthening could be observed. Here a better relaxation is obtained than in the previous curve, but it is still not complete, and we may conclude from this series of tests that cysteine can not carry out the function of the second relaxation factor.

(c) As reported in the previous paper (3), pH plays an important role in EDTA relaxation. At pH above 7.5 relaxation by EDTA hardly takes place, and a subsequent exchange of the high pH relaxation medium for one at lower pH often failed to cause complete relaxation, though some additional relaxation always occurred (see Fig. 8, Curve a). When contraction and relaxation are both carried out at pH below 6.0 (50 mM per liter veronal-acetate buffer) a complete relaxation is always obtained, but contraction itself is small as in Curves b_1 and b_2 of Figure 8. Now in the experiment of Fig. 8c the fiber contracted nicely more thah 50 per cent, and relaxed considerably at pH 6.3. Subsequent exchange of this relaxation medium for one of even lower pH (5.8) produced nearly complete relaxation. (Complete relaxation is sometimes not obtained under these conditions). Finally, in Curve d, when 2 mM per liter cysteine was added to the contraction medium (pH 6.3) and 20 mM per liter cysteine to the relaxation medium (pH 5.8, 8mM per liter Mg^{++}), rapid and complete relaxation occurred. In our "old, well-washed" fibers (more than 60 days in glycerol-water, and about 20 hours 0° washing in KCl aq.) these conditions consistently produced complete and rapid relaxation. However, when one of the fibers was taken through another cycle, the

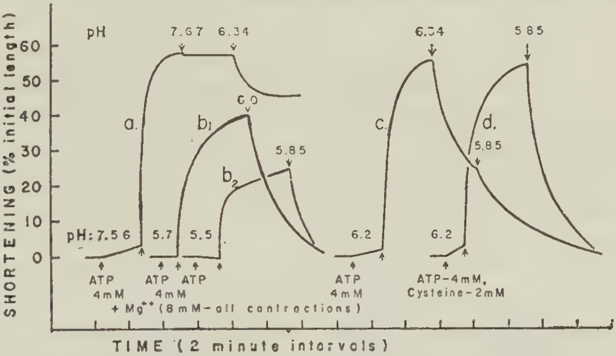

FIG. 8. The effect of pH changes and of cysteine at low pH. The pH of the contraction medium (adjusted with veronal-acetate buffer) is indicated on the left of each curve at the start of the contraction: that of the relaxation medium is shown at the top of each curve above the arrow indicating exchange of media. Each contraction was initiated by addition of 8 mM per liter of Mg^{++} (at the second upward arrow). The relaxation medium for Curves a, b and c contained 4 mM per liter, each of ATP Mg^{++} and EDTA; for curve d it contained 4 mM of ATP and EDTA, 8 mM of Mg^{++} and 20 mM cysteine per liter.

second contration was always smaller than the first, and the second relaxation slower than the first one. To sum up, cysteine and lower pH help the "old" fiber to relax completely, but this always seems to involve an irreversible lengthening by cysteine.

6. *Effects of Some Drugs and Inhibitors*—The effects of the enzyme inhibitors 2, 4-dinitrophenol (DNP) and potassium fluoride (KF) were studied in the experiments shown in Curves a and be of Fig. 9. Comparison with the dotted control curves indicates that these substances have a slight inhibitory effect on contraction but no effect on EDTA relaxation. Arsenate (Curve c) produces some lengthening of a contracted fiber, but does not influence EDTA relaxation, while 10 mM per liter arsenite (Curve d) has no effect on the contracted fiber, but may have a slight inhibitory effect on the relaxation process.

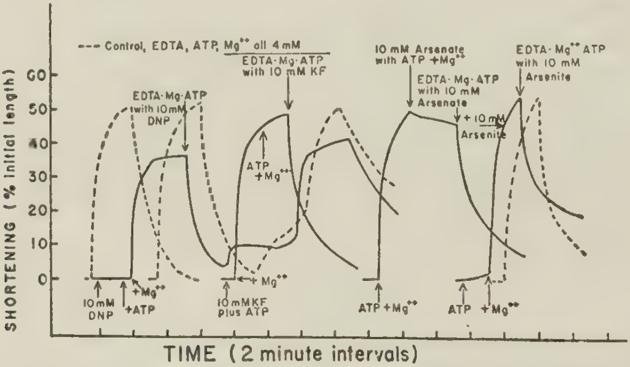

FIG. 9. Effects of some enzyme inhibitors. The dotted curves are controls in the standard contraction and EDTA relaxation media. Deviations from these conditions in the other cases are as indicated along each curve.

It is also observed that neither 10 mM per liter histamine, nor 20 mM per liter acetyl choline bromide, has a significant effect on contraction, relaxation, or response to Ca^{++} of our standard fibers.

7. *Glycerol-Treated Molluscan Smooth Muscle*—Glycerinated fibers from the anterior byssal retractor muscle of Mytilus edulis were prepared by the same standard procedure which was used for rabbit psoas fibers. Although the fibers of this muscle are unstriated, they could be prepared and used in this manner because, unlike mammalian smooth muscle, most of the cells extend nearly parallel and straight with little change in diameter almost the whole length of muscle (up to 5 cm.)

Small bundles of these fibers in 160 mM per liter KCl will shorten on application of ATP and Mg^{++} (4 mM per liter) comparably with rabbit muscle but a slower rate. They are also similar to rabbit fibers in that Mg^{++} in the shortening medium can be replaced with Mn^{++} but not with Ca^{++}.

EDTA has only a very weak relaxing effect on Mytilus fibers, suggesting that "standard" Mytilus fibers are comparable with "old and well-washed" psoas fibers. However, when EDTA relaxation in observed, it can be reversed by application of Ca^{++} or Mn^{++} just as in rabbit fibers.

In general our results with Mytilus fibers support the view that the basic mechanisms of the contraction cycle may be the same in invertebrate smooth muscle as in mammalian striated muscle.

DISCUSSION

The first experiments described above indicate that there are some differences between the relaxation effects of pyrophosphate and those the other relaxing factors (EDTA, myokinase, and the creatine kinase system), especially since under our conditions we failed to observe the accelerating effect of ATP on pyrophosphate relaxation reported previously (*1*) (*5*). Nevertheless, with respect to the important property of the absolute specificity of its requirement for Mg^{++}, pyrophosphate can still be classified with the above-mentioned "first" group of relaxation factors, whose high affinity for Mg^{++} has been assumed to be of major importance in their relaxing function.

On the other hand, the bivalent cation requirement of the cysteine lengthening process is not specific for Mg^{++}. The effect of cysteine on glycerol-treated fibers has been found to be qualitatively different from that of other relaxation factors in other respects, also. For example, (a) ATP is not needed for lengthening by cysteine, (b) some irreversible process has always been involved in the cysteine lengthening so far observed, and (c) cysteine plus Mg^{++} may produce (as in Fig. 7, b and c) a lengthening beyond the maximum obtainable with the EDTA relaxation medium.

The foregoing observations on on the effects of cysteine, mono-iodoacetate (IAA), and EDTA can be speculatively interpreted if it is assumed, in keeping with the suggestions of Kielley *et al.* (*7*) or Straub *et al.* (*8*), that there exist in the contractile protein two types of sulfur groups, one type (C) involved in the contractile process, and the other type (L) involved in the lengthening process. It would appear that the lengthening effect of cysteine is not primarily on the contractile units, but on L-type sulfur groups which are members of cross-links that hold together the contractile units. This is suggested because cysteine can lengthen a contracted, loaded fiber well beynd its untreated length, and because a fiber once contracted and then lengthened by cysteine cannot be washed and again made to cantract on addition of Mg^{++} and ATP.

These L-type sulfur groups could be disulfide linkages subject to reduction by cysteine, or they could be electrostatic attraction in which the negative member is an R-S$^-$ of the protein and is therefore competitively displaceable by cysteine; in either case, any divalent cation might facilitate the approach of the cysteine to a negatively charged proteine. The facts that cysteine takes effect rather rapidly (Fig. 3a) and (at least in the absence of Mg^{++}) that it is easy to remove by mere washing (Fig. 2a) somewhat favour displacement over reduction.

Among its other effects, IAA can halt cysteine lengthening, so presumably it can somehow restore cross-links at the L-sites. This restoration is probably not achieved by mere combination of IAA with free cysteine because washing away the free cysteine does not halt the lengthening so precipitously (Fig. 3b, 3c) and because IAA can halt the lengthening at a concentration which is roughly one-third that of the cysteine (Fig. 3a). It is also hard to see how IAA could restore *pisulfide* cross-links previously reduced by cysteine. On the other hand, had the original L-type groups been R-S$^-$ groups (later displaced by cysteine), it is at least conceivable that upon conversion to R-SCHCOO$^-$ (by IAA) they might regain the upper hand, perhaps because of increased average negativity or perhaps for steric reasons, over cysteine in competition for the hypothetical positively charged member of the cross-link.

One observation (Fig. 3a) seems at variance with the foregoing speculation. A fiber once contracted by Mg^{++} plus ATP stays contracted indefinitely unless treated with a reagent, *e.g.*, a relaxation factor, that reacts with the contractile units. We have just proposed that cysteine does not react with the contractile units, so that after the effect of cysteine is halted by IAA we should expect the contractile units to be still in the contracted state. Yet the latter part of Fig. 3a shows that the fiber contracted in response to a second addition of Mg^{++}+ATP. This observation has at least three alternative explanations, none of which can be excluded on the basis of our present information: (1) Contrary to the foregoing speculation, cysteine might also have reacted at the C-type sites, displacing the first does of ATP+Mg^{++}, (2) The second does of Mg^{++}+ATP might have reached a deeper, yet unstimulated core of the fiber (it is cogent that contraction was not complete), (3) IAA somehow fortifies the effect of Mg^{++}+ATP, by opening up additional contractile sites.

The observations of Fig. 4 show that IAA must also act at the C-sites because it interferes with EDTA relaxation, a phenomenon presumed to occur at the C-sites. If the relaxing action of EDTA is, in fact, due to its chelation of bound magnesium away from an R-S$^-$ group of the protein (*7*) we can suppose, to complete our speculation, that the conversion of the C-type R-S$^-$ to R-SHCOO$^-$ if anything improves the role of this group in contraction, and enables it to compete successfully with EDTA for the bound Mg^{++}.

When old (60 days) and well washed (more than 10 hours) fibers are allowed to shorten maximally, then cannot be completely relaxed by EDTA. This behavior supports the idea that there exists a "second factor" (*6*) which is removed by long storage and washing, and which is necessary for complete relaxation. However, when the shortening of such old well-washed fiber is limited to the physiological range (30-35 per cent) of initial length the standard EDTA medium will repeatedly produce complete relaxation (Fig. 6). Moreover, DNP (and to a lesser degree KF) limits the amount of shortening to about 35 per cent without reducing the initial rate. These two independent results, and also the frequently observed fairly sharp change in rate between the earlier and later phases of shortening, all suggest that there are two qualitatively different phases in complete shortening: (a) the first one is represented by the first rapid phase of the shortening (up to 30-35 per cent of initial length) which is stopped and reversed by EDTA, probably by formation of a "4 species complex" of EDTA, Mg^{++}, ATP and the contractile protein (*3*); (b) the slow second phase of the shortening is produced when the "second relaxation factor" (*6*) is absent and can not be reversed by either EDTA or other factors of the "first" group.

SUMMARY

1. Neither calcium nor manganese can take the place of magnesium is supplying the divalent ion requirement for pyrophosphate relaxation of glycerol-treated muscle fibers; in this respect pyrophosphate is similar to other relaxation factors of the "first" group: myokinase, the creatine-kinase system, and EDTA.

2. Cysteine causes a lengthening of contracted glycerinated fibers, which is accelerated by any one of these bivalent cations (Mg^{++}, Ca^{++} or Mn^{++}).

The lengthening caused by cysteine is also qualitatively different in other respects from that by other relaxation factors (including pyrophosphate), and may be additive to relaxations produced by them.

S. Mono-iodoacetate has a slight acclerating effect on shortening in the ATP$+$Mg^{++} medium, it can stop cysteine lengthening, and it causes subsequent relaxation by the EDTA relaxation medium to be incomplete.

4. Old and well-washed glycerinated fibers (more than 60 days in 50 per cent glycerol-water at $-10°$ and more than 10 hours washing in 160 mM per liter KCl at 0°) relax only incompletely in the EDTA medium; this supports Ebashi's idea (*6*) that there is a seconp relaxation factor which is removed by the long preparation procedures.

5. The old well-washed fibers just described can be made to relax completely in the presence of cysteine and lower pH. However, some irreversible process in involved in such lengthening, which indicates that cysteine cannot function as the second relaxation factor.

6. DNP, KF, arsenate and arsenite have various but small inhibitory

effects on the amount of shortening, but none on relaxation by EDTA.

We wish particularly to thank Dr. M.F. Morales (Naval Medical Research Institute, Bethesda, Maryland, U.S.A.) for many valuable suggestions and criticisms.

REFERENCES

(*1*) Bozler, E., *J. Gen. Physiol.* **38**, 149 (1954)
(*2*) Watanabe, S., *Arch. Biochem. Biophys.* **54**, 559 (1955)
(*3*) Wataname, S. and Sleator, W., *Arch. Biochem. Biophys.*, **68**, 81 (1957)
(*4*) Weber, H.H., *Proc. Roy. Soc.* B, **139**, 512 (1952)
(*5*) Bendall, J.R., *Nature*, **172**, 586 (1953)
(*6*) Kumagai, H., Ebashi, S. and Takeda, F., *Natuae*, **176**, 166 (1955)
(*7*) Kielley, W.W. and Bradley, L.B., *J. Biol. Chem.*, **218**, 653 (1956)
(*8*) Straub, W. and Turba, F., *Biochem. Z.* **327**, 473 (1956)
(*9*) Friess, E.T., Morales, M.F. and Bowen, W.J., *Arch. Biochem. Biophys.* **53**, 311 (1954)

The Journal of Biochemistry, Vol. 45, No. 10, 1958

OXIDATION OF TRIPEPTIDES CONTAINING HYDROXYAMINO ACID RESIDUE BY PERIODIC ACID

By SETSURO FUJII and KIKUO ARAKAWA

*(From the Department of Medical Chemistry and the 3rd
Department of Internal Medicine, Factulty of
Medicine, Kyushu University, Fukuoka)*

(Received for publication, November 29, 1957)

Some experiments of treating proteins with periodic acid have been tried by some investigators (1--5), but a little has been known about the details of its reaction mechanism.

In our previous reports, we have investigated the action of periodic acid on amino acids (*6*) and dipeptides (*7*) to confirm the existence of an analogous oxidative action on either hydroxyamino acids and hydroxyamino acid residue contained in peptide. According to the results, not only hydroxyamino acid but also non-hydroxyamino acids were oxidized by periodic acid at higher temperature. But dipeptides containing hydroxyamino acid residue were more rapid in its oxidation velocities than that without the residue.

Present communication deals with the oxidation velocities of tripeptides by periodic acid comparing with that of dipeptides reported previously.

EXPERIMENTAL

I. Synthesis of Materials

Only new peptides among the synthesized are described below as to the details. And all of them were confirmed to have only one spot respectively by paperchromatography before they were used in oxidation experiment.

DL-Serylglycylglycine was synthesized by azide method. As carbobenzoxy-DL-serylglycine hydrazide was not obtainable by the reaction of carbobenzoxy-DL-serylglycine benzylester (*7*) and hydrazine hydrate, it was restarted from its methylester.

*Carbobenzoxy-*DL*-Serylglycine Methyl Ester*; m.p., 91-93°
$C_{14}H_{18}O_6N_2$ (310.30) Calculated: N 9.03
Found: ,, 8.97

*Carbobenzoxy-*DL*-Seryglycine Hydrazide*; m.p., 189°
$C_{13}H_{18}O_5N_4$ (310.31) Calculated: N 18.08
Found: ,, 18.21

*Carbobenzoxy-*DL*-Serylglycylycine Benzyl Ester*; m.p., 134°
$C_{22}H_{25}O_7N_3$ (443.44) Calculated: N 9.69
Found: ,, 9.65

DL-*Serylglycylglycine*; m.p (decomposition), 185-188°

$C_7H_{13}O_5N_3$ (219.20) Calculated: N 19.17
Found: ,, 19.08

Clycyl-DL-serylglycine was also synthesized by the azide method.

*Carbobenzoxy-Glycyl-*DL*-Serine Methyl Ester*; m.p., 63°

$C_{14}H_{18}O_6N_2$ (310.30) Calculated: N 9.03
Found: ,, 9.11

*Carbobenzoxy-Glycyl-*DL*-Serine Hydrazide*; m.p., 183°

$C_{13}H_{18}O_5N_4$ (310.31) Calculated: N 18.04
Found: ,, 18.21

*Carbobenzoxy-Glycyl-*DL*-Serylglycine Benzyl Ester*; m.p., 161°

$C_{22}H_{25}O_7N_3$ (443.44) Calculated: N 9.69
Found: ,, 9.39

*Clycyl-*DL*-Serylglycine*; m.p., 201°

$C_7H_{13}O_5N_3$ (219.20) Calculated: N 19.15
Found: ,, 18.96

Glycyl-DL-threonylglycine was synthesized by the mixed anhydride method (*8*).

DL-*Threonine Ethyl Ester Hydrochloride*—4 g. of DL--threonine was suspended in 40 fold of ethanol and saturated with dry hydrogen chloride at room temperature. After standing overnight at 0°, ethanol and hydrogen chloride were evaporated. Crystalline needle were obtained and recrystallized from ethanol-ether, yield 4.8 g., m.p. 119°.

$C_6H_{14}O_3Cl$ (183.64) Calculated: N 19.30
Found: ,, 19.13

*Carbobenzoxy-Glycyl-*DL*-Threonine Ethyl Ester*—To 3.4 g. of carbobenzoxyglycine and 1.64 g of triethylamine dissolved in 30 ml. of toluene was added 2.22 g. of *iso*-butylchlorocarbonate under the stirring and cooling at −5°. After 5 minutes, 3 g. of DL-threonine ethyl ester hydrochloride and 1.64 g. of triethylamine dissolved in 25 ml. chloroform was added to the mixture and stood for 2 hours at 0° and overnight at 8°. The mixture was washed with water and bicarbonate solution and then dried over sodium sulfate. As it began to be turbid during the drying, it was evaporated after 4 hours and added petroleum ether, 2.1 g. of the precipitate melted at 98°.

$C_{16}H_{22}O_6N_2$ (338.35) Calculated: N 8.28
Found: ,, 8.25

*Carbobenzoxy-Glycyl-*DL*-Threnine Hydrazide*; m.p., 182°

$C_{14}H_{20}O_5N_4$ (324.33) Calculated: N 17.28
Found: ,, 17.44

*Carbobenzoxy-Glycyl-*DL*-Threonylglycine Benzyl Ester*; m.p., 137°

$C_{23}H_{27}O_7N_3$ (457.47) Calculated: N 9.19
Found: ,, 9.11

*Glycyl-*DL*-Threonylglycine Monohydrate*; m.p., (decomposition), 214–216°

		N	C	H
$C_8H_{17}O_6N_3$ (251.24)	Calculated:	16.73	38.20	6.81
	Found:	16.77	38.03	6.60

Glycylglycyl-DL-threonine was synthesized by the use of *N*, *N'*-dicyclohexylcarbodiimide (*9*).

Carbobenzoxy-Glycylglycyl-DL-*Thereonine Ethyl Ester*—To the mixture of 0.00865 moles of carbobenzoxyglycylglycine, dicyclohexylcarbodiimide and 50 ml. of methylene chloride was added methylene chloride solution of 0.00865 moles-DL-threonine ethyl ester hydrochloride and triethylamine. After standing overnight, the precipitate was filtered off and filtrate was washed and dried over sodium sulfate. The solution was then evaporated and the residue was dissolved in small amount of ethyl acetate and the undissolved was filtered off. After addition of petroleum ether, precipitate was recrystalized from ethyl acetate and petroleum ether three times and melted at 119–120°. Yield 2.1 g.

$C_{18}H_{25}O_7N_3$ (395.40) Calculated: N 10.63
Found: ,, 10.60

Carbobenzoxy-Glycylglycyl-DL-*Threonine*—0.005 moles of carbobenzoxy-glycylglycpl-DL-threonine ethylester dissolved in 40 ml. of methanol was treated with equivalent amount of 2 N sodium hydroxide solution for thirty minutes at room temperature. After the acidification with hydrochloric acide, the precipitate was washed with cold water. Yield 1.4 g., m.p., 196°.

$C_{16}H_{21}O_7N_3$ (367.35) Calculated: N 11.44
Found: ,, 11.38

Glycylglycyl-DL-*Threonine Monohydrate*—After the hydrogenaton of the above in the mixture of glacial acetic acide, water and methanal with hydrogen gas and palladium black, the product was obtained in the technique as usual and melted at 150–154° (decomposition). Yield 0.6 g.

		N	C	H
$C_8H_7O_6N_3$ (251.24)	Calculated:	16.73	38.2	6.81
	Found:	16.45	27.8	6.73

II. Oxidation

Each 10 mg. of material was oxidized with 1 ml. of 0.5 M HIO_4, prepared from KIO_4 according to Jackson *et al.* (*10*), in a medium neutralized by addition of alkaline phosphate buffer. Oxidation velocity of tripeptide was determined by mesuring ammonia liberated from them in the same technique as our previous reports (*6*, *7*).

RESULTS AND DISCUSSION

Among the tripeptides investigated, only DL-serylglycylglycine was oxidized appreciably at room temperature as shown in Fig. 1. The curve shows that the ammonia liberation from it becomes suddenly flat as soon as it reached one third of the amount from the total N. It was also disclosed by paperchromatography after hydrolysis by hydrochloric acid that the reaction mixture oxidized for 3 hours at room temperature contained no more any serine but glycine. It is evident from these results that the serine residue of DL-serylglycylglycine is almost selectively oxidizable by the action of periodic acid.

On the contrary, other tripeptides were hardly oxidized at 15° and a higher temperature was needed to liberate ammonia from them as shown in Fig. 2. In those cases, however, it was disclosed by paperchromatography that the hydroxyamino acid residue was still remained in the reaction mixture oxidized at 60° even after the liberation of ammonia in the amount of one third calculated from the total N. Among them, glycylglycyl-DL-threonine, which had hydroxyamino acid residue in its C-terminal, was most resistant to periodic acid. Because the curve of it is slacker than that of previously reported glycyl-DL-threonine or glycyl-DL-serine (*8*), it is conceivable that the longer the chain of other residue combined with the amino group of hydroxyamino acid residue the more resistant to periodic acid.

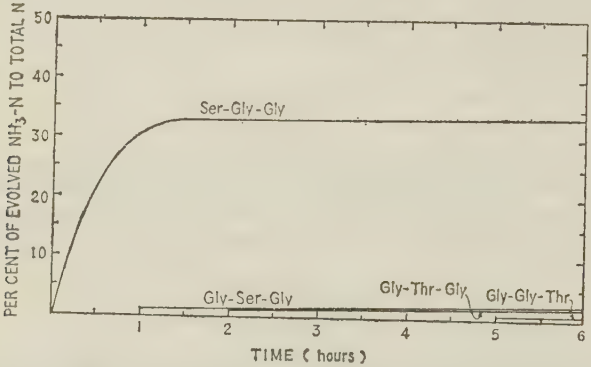

FIG. 1. Oxidation velocities of tripeptides by the action of neutral periodate at 15°.

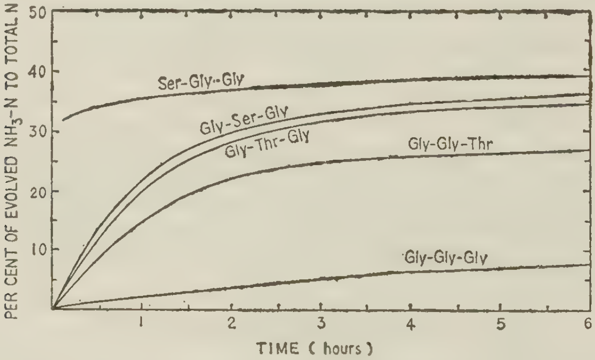

FIG. 2. Oxidation velocities of tripeptides by the action of neutral periodate at 60°.

10 mg. of material dissolved in 1 ml. of water and 20 ml. of $M/25$ phosphate buffer of pH 8.03 was oxidized with 1 ml. of $0.5\,M$ HIO_4. After the oxidation, excess of HIO_4 was removed as barium salt. The filtrate and washings were combined and NH_3 from them was distilled and determined by the use of Parnas' apparatus.

Glycyl-DL-serylglycine and glycyl-DL-threonylglycine containing hydroxyamino acid residue in the middle were oxidized in almost equal velocities to those of glycyl-DL-serine and those of glycyl-DL-threonine report ed previously. This fact may suggest that other residue combined with carboxyl group of hydroxyamino acid residue do not inhibit the oxidation of tripeptide, and most of the inhibition is derived from other residue combined with amino gronp of hydroxyamino acid residue.

SUMMARY

1. Some new tripeptides were synthesized and oxidized by periodic acid in a neutral medium.

2. For the selective oxidation of hydroxyamino acid residue in tripeptide, its amino group must be free.

3. The length of the other residue combined with the amino group of a hydroxyamino acid residue was inverse to the oxidizability of the peptide.

4. Free or not free of its carboxyl group of a hydroxyamino acid residue was independent to the oxidizability of the peptide.

The authors are indebted to Dr. R. Hirohata for his valuable advices and encouragement.

REFERENCES

(*1*) Desnuelle, P., Antonin, S., and Casal, A., *Bull. soc. chim. biol.*, **29**, 694 (1947)
(*2*) Goebel, W.F., and Perlmann, G.E., *J. Exp. Med.*, **89**, 479 (1949)
(*3*) Jansen, E.F., Curl, A.L., and Balls, A.K., *J. Biol. Chem.*, **189**, 671 (1951)
(*4*) Hamada, M., and Maekawa, K., *Sci. Bull. Fac. Agr. Kyushu Univ.*, **13**, 180 (1951)
(*5*) Maekawa, K., and Kushibe, M., *Bull. Chem. Soc. Japan*, **27**, 277 (1954)
(*6*) Arakawa, K., *J. Biochem.*, **44**, 217 (1957)
(*7*) Fujii, S., Arakawa, K., and Aoyagi, N., *J. Biochem.*, **44**, 471 (1957)
(*8*) Vaughan, J.R., and Osato, R.L., *J. Am. Chem. Soc.*, **73**, 5553 (1951)
(*9*) Sheehan, J.C., Goodman, M. and Hess, J.P., *J. Am. Chem. Soc.*, **78**, 1367 (1956)
(*10*) Jackson, E.L., and Hudson, C.S., *J. Am. Chem. Soc.*, **59**, 994 (1937)

The Journal of Biochemistry, Vol. 45, No. 10, 1958

TWO TYPES OF HYDROQUINONE OXIDASE OF PSEUDOMONAS AERUGINOSA

By TANEAKI HIGASHI

(*From the Department of Biology, Faculty of Science, University of Osaka, Osaka*)

(Received for publication, June 26,1958)

In an earlier investigation on bacterial cytochromes in Okunuki's laboratory, Horio (*1*) devised a simple continuous procedure for the high purification of respiratory proteins from *Pseudomonas aeruginosa: Pseudomonas* cytochrome$_{554}$ (P-cytochrome$_{554}$), P-cytochryme$_{554}$, P-cytochrome$_{GB}$ and P-blue protein. By further purification of P-cytochrome$_{GB}$, Horio, Higashi, Okunuki *et al.* (*2*, *3*) demonstrated that P-cytochrome$_{GB}$ functions as a cytochrome oxidase in living cells of *Pseudomonas aeruginosa.* They showed that it has a complex absorption spectrum as if it contained cytochrome a_2 and two other cytochrome c-like cytochrome in a conjugated state. It was named P-cytochrome oxidase, since it could rapidly oxidize reduced P-cytochrome$_{551}$, reduced P-blue protein, and hydroquinone. The oxidation occurred without addition of any cofactor. During purification of P-cytochrome oxidase, using hydroquinone as substrate, it was noticed that the more highly the oxidase was purified, the more strongly was its activity inhibited by potassium cyanide and carbon monoxide. This phenomenon might have been caused by the following situations: 1) The cell-free extract of *Pseudomonas aeruginosa* contained a substance having an affinity for cyanide and carbon monoxide. During purification this was removed and the cyanide and carbon monoxide then attacked the oxidase more severely. or 2) Acyanide- and carbon monoxide- insensitive enzyme capable of oxidizing hydroquinone was present in the initial cell-free extract.

To study these possibilities, the purification procedure of P-cytochrome oxidase (*1*) was re-examined. This study proved that in addition to P-cytochrome oxidase *Pseudomonas aeruginosa* has an enzyme which can oxidize hydroquinone and which is remarkably less sensitive to cyanide and carbon monoxide. This enzyme was partially purified in a state completely separate from P-cytochrome oxidase, and was tentatively named P-hydroquinone oxidase. The physical and physiological differences between P-cytochrome oxidase and P-hydroquinone oxidase were compared.

MATERIALS AND METHODS

Pseudomonas aeruginosa was grown in submerged culture at 37° in a liquid medium containing nitrate as described in the previous report (*1*). The cells were harvested and their acetone powder was prepared.

P-cytochrome$_{554}$, P-cytochrome$_{551}$, P-blue protein and P-cytochrome oxidase were purified from the acetone powder according to the method of Horio, Higashi, Okunuki *et al.* (*1*, *3*). Native, yeast cytochrome c was crystallized from baker's yeast according to the method of Nozaki, Horio, Okunuki *et al.* (*4–6*).

Oxygen consumption was measured at 30° by Warburg manometer under the following conditions, unless indicated otherwise in the text: Main chamber, 1.0 ml. of enzyme, 0.4 ml. of 0.2 *M* phosphate buffer (pH 6.4) and distilled water to a final volume of 1.8 ml. Side chamber, 0.2 ml. af 0.5 *M* substrate. The reaction was started after a 5 minute-equilibration period.

Optical densities were measured using a Shimazu photoelectric spectrophotometer, type QB-50, at room temperature.

The dry weight of protein in the solution was estimated as follows: 3 volumes of 0.1 *M* trichloroacetic acid was added to a part of the solution and the mixture centrifuged. The resulting precipitate was washed three times with 0.001 *M* trichloroacetic acid. The washed precipitate was then dried at 105° for 12 hours and weighed.

Zone-electrophoresis was carried out on a column os starch (3 cm. in diameter, 30 cm. in height) in 0.1 *M* sodium phosphate buffer of pH 7.0 in a refrigerator (5–6°) with frequent adjustment of amperage to 20 mA (330–370 v).

Diphosphopyridine nucleotide (DPN) was prepared from baker's yeast by the method of Okunuki *et al.* (*7*) and triphosphopyridide nucleotide (TPN) from sheep liver by the method of Horecker and Kornberg (*8*).

RESULTS

Extraction and Purification of p-Hydroquinone Oxidase—The acetone powder of *Pseudomonas aeruginosa* cells which has been grown in 100 liters of the liquid medium was homogenized with the volumes of 0.1 *M* citrate buffer of pH 7.2. The mixture was heated to 40° in water bath and maintained at this temperature for 10 minutes with continuous stirring. It was then cooled to room temperature under running tap water. The solution was filtered on a Büchner funnel with the aid of Hyflo Super Cel. This extraction procedure was repeated twice. The filtrates were combined and 3 per cent rivanol solution added until a fibrous precipitate no longer formed. After filtration, the clear brown filtrate was fractionated with ammonium sulfate. The fraction precipitated between 30 and 95 per cent-saturation was collected and dialyzed for 24 hours against running tap water at below 5°. After centrifugation, the solution was passed through a column of Duolite CS-101 (100–105 mesh) which had been buffered with 0.1 *M* ammonium phosphate buffer to pH 5.8 and washed with distilled water. P-cytochrome oxidase present in the solution was retained on the colum and reserved for its further purification. The passed solution, now free from P-cytochrome oxidase, was passed through a column of activated aluminium oxide (approximately 300 mesh) which had been buffered to pH 6.0 with 0.1 *M* ammonium phosphate buffer and washed with distilled water. This activated aluminium oxide adsorbed P-cytochrome$_{554}$, P-cytochrome$_{551}$, and P-blue protein. Therefore, the resulting solution contained no P-cytochrome oxidase, P-cytochrome$_{554}$, P-cytochrome$_{551}$ or P-blue protein and did not show any cytochrome-like absorption shectrum after reduction with dithionite. Although free from P-cytochrome oxidase, the solution oxidized hydroquinone as rapidly as P-cytochrome oxidase did. The enzyme present which oxidized hydroquinone, but whose true function was unknown was tentatively called P-hydroquinone oxidase. Table I givesa summary of the purification procedure of P-hydroquinone oxidase. As described in the previous reports (*2*, *3*), P-cytochrome oxidase was completely inhibited in the presence of a final concentration of 10^{-3} *M* of cyanide. Therefore, based an assumption that hydroquinone oxidation insensitive to the cyanide was caused by P-hydroquinone oxidase, calculations were performed and the results are presented in the Table. P-hydroquinone oxidase was fractionated between 50 and 75 per cent of acetone and between 50 and 65 per cent-saturation of ammonium sulfate. Zoneelectro phoreis of this enzyme could rise up its specific activity from 1.5 to 2times.

TABLE I

Summary of Purification Procedures of Pseudomonas Hydroquinone Oxidase from Pseudomonas aeruginosa

Specific activity was defined as μl. of oxygen consumed during initial 10 minutes per 10 mg. dry weight of protein.

Purification step	Specific activity	Yield (%)
Extract from acetone powder	19.3	100
Eluate from CS-101	34.9	16.7
Eluate from alumina column	214.0	16.7
After zone-electrophoresis	358.0	11.1

TABLE II

Stability of Pseudomonas Hydroquinone Oxidase to Heat Treatment

The preparations were maintained 10 minutes at each temperature.

Temperature	Relative activity (%)*
—	100
50°	95
60°	74
70°	14
100°	10

* Relative activity was calculated from the activity during the initial 10 minutes of the reaction.

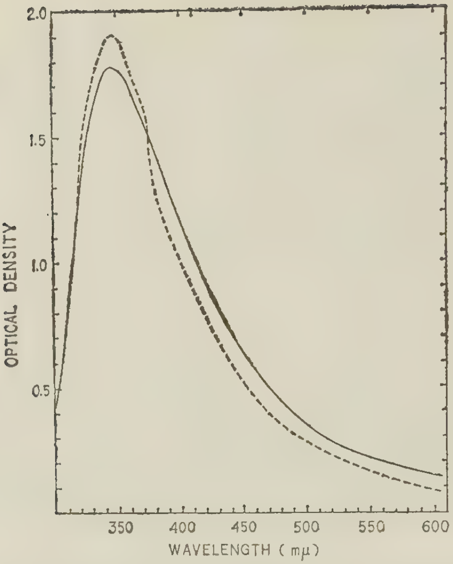

Fig. 1. Absorption spectrum of most purified preparation of *Pseudomonas* hydroquinone oxidase.

Solid line, oxidized form; broken line, dithionite-reduced form.

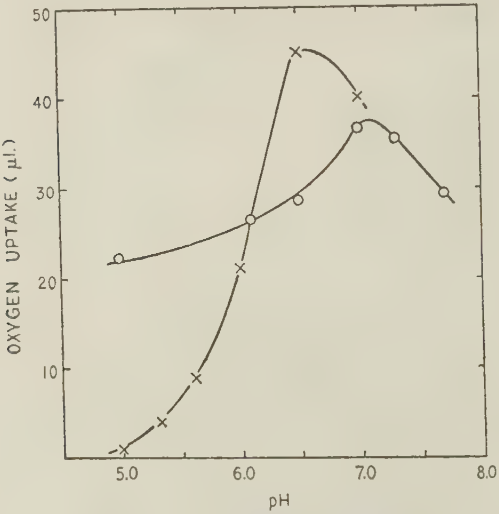

Fig. 2. Effect of pH on the hydroquinone oxidation of *Pseudomonas* hydroquinone oxidase and *Pseudomonas* cytochrome oxidase.

Phosphate buffer. Activities expressed as μl oxygen consumed during initial 10 minutes. Above pH 7, hydroquinone is rapidly autoxidizable, and the values plotted represent the difference between oxygen consumption in the presence and absence of enzyme.

—×—, P-hydroquinone oxidase; —○—, P-cytochrome oxidase

Stability of P-Hydroquinone Oxidase to Heat, Dialysis, and Lyophilization—In the final stage of purification achieved, P-hydroquinone oxidase lost almost all of its activity when heated at 70° for 10 minutes, but retained most of its activity after being maintained at 60° for 10 minutes, as shown in Table II. The oxidase could not be appreciably purified by heat treatment, however. The preparation was fairly stable when dialyzed against distilled water at room temperature (*ca.* 15°) for 24 hours. When lyophilized and stored in a dry state at 0–5° for one week, the total activity of the preparation was retained.

Absorption Spectrum of the Purest Preparation of P-Hydroquinone Oxidase—The preparation of P-hydroquinone oxidase at the final stage of purification was yellowish brown, and unlike P-cytochrome oxidase (3) did not show any cytochrome-like absorption spectum. There was a strong absorption peak at about 350 mμ (Fig. 1). This peak did not change appreciably on addition of a fairly large amount of sodium dithionite, though the difference in the spectra in the presence and absence of sodium dithionite showed a slight peak around 350 mμ, a shoulder around 370 mμ and a slight minimum at about 400 mμ.

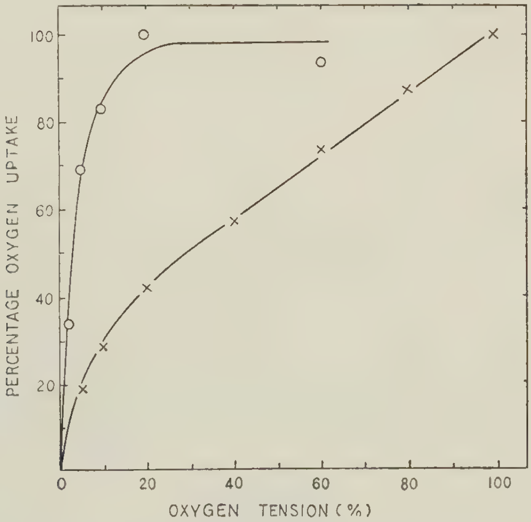

Fig. 3. Effect of oxygen tension upon the rates of hydroquinone oxidation by *Pseudomonas* hydroquinone oxidase and *Pseudomonas* cytochrome oxidase.

Maximal rate of oxygen consumption during the first 10 minutes of reaction was denoted as 100 per cent.

—×—, P-hydroquinone oxidase; —○—, P-cytochrome oxidase.

pH Optimum of P-Hydroquinone Oxidase—As shown in Fig. 2, P-hydroquinone oxidase oxidizes hydroquinone most rapidly at about pH 6.5.

Effect of Oxygen Tension on the Activity of P-Hydroquinone Oxidase—As shown in Fig. 3, hydroquinone oxidation by P-hydroquinone oxidase increases with increased oxygentension.

Effect of Potassium Cyanide and Carbon Monoxide on P-Hydroquinone Oxidase—The oxidation of hydroquinone by P-hydroquinone oxidase was scarcely affected by potassium cyanide or carbon monoxide as a concentration sufficient to inhibit the activity of P-cytochrome oxidase completely (Table III).

Table III

Inffuence of Carbon Monoxide and Cyanide on Pseudomonas Hydroquinone Oxidase and Pseudomonas Cytochrome Oxidase

Components of gas phase (%)			Light	Inhibition of oxygen uptake (%)	
				P-hydroquinone oxidase	P-cytochrome oxidrse (3)
O_2	CO	N_2			
10	90		−	15	94
			+	12	93
20	80		−	0	—
			+	0	—
10	20	70	−	—	94
			+	—	44
Final concentration of cyanide (*M*)					
10^{-6}				—	29
10^{-5}				—	96
10^{-4}				—	100
10^{-3}				0	100

Influences of carbon monoxide were examined at 25° and those of cyanide at 30°.

Table IV

Specificity of Pseudomonas Hydroquinone Oxidase to Various Chemical Reductants

Substrate	Relative activity (%)	
	P-hydroquinone oxidase	P-cytochrome oxidase (*3*)
Hydroquinone	100	100
p-Phenylenediamine	23	92
Pyrocatechol	0	34
L-Ascorbic acid	45	222

Substrate-Specificity of P-Hydroquinone Oxidase—Substrate-specificity of P-hydroquinone oxidase was compared with that of P-cytochrome oxidase. As shown in Table IV, of the four substrates tested (hydroquinone, *p*-phenylenediamine, L-ascorbate and pyrocatechol) P-hydroquinone oxidized hydroquinone most rapidly, while P-cytochrome oxidase oxidized L-ascorbate twice as fast as hydroquinone.

P-hydroquinone oxidase could not oxidize the following substances: reduced P-cytochrome$_{554}$, P-cytochrome$_{551}$, P-blue protein, yeast cytochrome c, or DPN. P-cytochrome oxidase however could rapidly oxidize reduced P-cytochrome$_{551}$ and P-blue protein, and slowly oxidized reduced yeast cytochrome c, though having little action on reduced P-cytochrome$_{554}$ (Table V).

TABLE V

Specificity of Pseudomonas Hydroquinone Oxidase to Various Biological Reductants

Oxidations were measured spectrophotometrically at room temperature.

Substrate	Relative activity of oxidation*	
	P-hydroquinone oxidase	P-cytochrome oxidase (*3*)
DPNH	—	
Reduced P-cytochrome$_{554}$	—	±
Reduced yeast cytochrome c	—	+
Reduced P-cytochrome$_{551}$	—	⧻
Reduced P-blue protein	—	⧻

* In rough comparison with one another.

Effect of Various Other Substances on the Oxidation of Hydroquinone by P-Hydroquinone Oxidase—Even after prolonged dialysis, the oxidation of hydroquinone by P-hydropuinone oxidase was hardly influenced by the following substances: flavine adenine dinucleotide, flavine mononucleotide, riboflavine, TPN, and DPN. None of the metals tested including Mo^{6+}, Fe^{+++}, Co^{++}, Zn^{++} and Cu^{++} (final concentration 1×10^{-4} *M*) had any effect on hydroquinone oxidation. The oxidation was not influenced by ethylene diamine tetraacetate, diethyldithiocarbamate, *p*-chloromercuribenzoate, or azide. Azide had been reported to be a strong inhibitor on the flavoproteincatalyzed respiration of *Candida albicans* which is not sensitive to cyanide or carbon monoxide (*9*).

DISCUSSION

In 1937, Yamagutchi (*10*) reported that cell-free extract of *Bacillus pyocyaneus* can oxidize *p*-phenylenediamine, *p*-aminophenol, and hydroquinone (activity decreasing in this order) as well as reduced mammalian cytochrome c. In contrast to the common concept of "cytochrome oxidase", his indophenol oxidase was not affected by a gas phase of 95 per cent carbon monoxide and 5 per cent oxygen, though the oxidation of each of the substrated mentioned above was strongly inhibited by a low concentration of cyanide (10^{-3} to 10^{-4} M). Horio, Higashi, Okunuki, *et al.* (*2*, *3*) have found that a cell-free extract of *Pseudomonas aeruginosa* can oxidize these chemical reductants. As with Yamagutchi's preparation oxidation by the *Pseudomonas* extract was inhibited by a low concentration of cyanide, but in contrast to Yamaguchi's preparation was strongly inhibited by carbon monoxide. This *Pseudomonas* extract could be easily and completely separated into five components; P-cytochrome oxidase, P-cytochrome$_{554}$, P-cytochrome$_{551}$, P-blue protein, and P-hydroquinone oxidase (*1*, *2*, *3*). Though both oxidase rapidly oxidize hydroquinone in the absence of added cofactors, there are notable differences between their properties: P-hydroquinone oxidase shows no cytochrome-like absorption peak, oxidizes neither reduced P-cytochrome$_{551}$, nor reduced P-blue protein, and is scarcely affected by cyanide or carbon monoxide. Moreover, hydryquinone oxidation by P-cytochrome oxidase reaches maximal rate at about 20 per cent of oxygen, while that by P-hydryquinone oxidase increases steadily up to 100 per cent of oxygen. This lower affinity for oxygen suggests that P-hydroquinone oxidase may contoin flavine. Further purification of the enzyme is, however, necessary to demonstrate whether this is so.

It is well known that *Pseudomonas aeruginosa* contains nitrate reductase as well as P-cytochrome oxidase and under suitable conditions forms nitrite reductase and pyocyanine. Therefore, metabolism can probably occur *via* the following three terminal oxidative pathways; 1) P-cytochrome oxidase, 2) nitrate and nitrate reductase, and 3) pyocyanine.

Using a strain of *Acetobacter* which does not produce pyocyanine, Nakayama* (personal communication) has concluded that oxidation by the resting cells of various substrates at an oxygen tension similar or air might be carried out, by at least two essentially different respiratory systems. Based on the observation that the respiration can be only partially inhibited by cyanide, he concluded that one system is sensitive to cyanide and the other not. On the other hand, Lenhoff, Nicholas and Kaplan (*11*) have reported that *Pseudomonas fluorescens* produces cytochrome oxidase only at a rather low oxygen tension and at higher oxygen tensions the socalled flavine respiration occurs. In *Pseudomonas aeruginosa* cells P-hydroquinone oxidase may function in a respiratory system other than the cytochrome system. At present the true *in vivo* function of P-hydroquinone oxidase is unknown.

---

* Nakayama, T., Sasada Shoten, Nada, Kobe, Japan.

SUMMARY

Pseudomonas aeruginosa contains besides P-cytochrome oxidase, an enzyme which can be extracted in a water-soluble state and which oxidizes hydroquinone, L-ascarbate and *p*-phenylenediamine (the rate decreasing in this order) without addition of any cofactor. This enzyme was purified about 19 fold starting from an extract of acetone-powder of the cells. The most purified preparation had no cytochrome-like absorption spectrum and could not oxidize reduced P-cytochrome$_{551}$ or reduced P-blue protein, the substrates which are oxidized by P-cytochrome oxidase. Unlike P-cytochrome oxidase, oxidation of hynroquinone by this enzyme is scarcely influenced by cyanide or carbon monoxide. Increase of oxygen tension up to 100 per cent results in a gradual increase in the oxidative rate.

This enzyme was tentatively named *Pseydomonas* hydroquinone oxidase, since hydroquinone was the most rapidly oxidized of the substrates tested and since its true biological function is not yet known.

The author would like to express his thanks to Prof. K. Okunuki and Dr. T. Horio far their valuable guidance. Thanks are also due to his colleagues Messrs. M. Nozaki, T. Yamanaka, J. Yamashita and H. Mizushima of the same laboratory for their help and discussion and Messrs. M. Nakai and K. Kusai of Amagasaki Factory of Nagase and Co., Ltd., for mass-culture of the microörganisms.

REFERENCES

(*1*) Horio, T., *J. Biochem.*, **45**, 195 (1958)
(*2*) Horio, T., *J. Biochem.*, **45**, 267 (1958)
(*3*) Horio., T., Higashi, T., Matsubara, H., Kusai, K., Nakai, M., and Okunuki, K., *Biochim. et Biophys. Acta*, **29**, 297 (1958)
(*4*) Nozaki, M., Yamanaka, T., Horio, T., and Okunuki, K., *J. Biochem.*, **44**, 453 (1957)
(*5*) Nozaki, M., Mizushima, H., Horio, T., and Okunuki, K., *J. Biochem.*, in press.
(*6*) Hagihara, B., Horio, T., Yamashita J., Nozaki, M., and Okunuki, K., *Nature*, **178**, 629 (1956)
(*7*) Okunuki, K., Hagihara, B., Sekuzu, I., Nozaki, M., and Nakai, M., *J. Biochem.*, **42**, 389 (1955)
(*8*) Horecker, B.L., and Kornberg, A., Methods in Enzymology, Academic Press Inc. Publishers, New York, Vol. III, 879 (1957)
(*9*) Ward, J.M., and Nickerson, W.J., *J. Gen. Physiol.* **41**, 703 (1958)
(*10*) Yamaguchi, S., *Acta Phytochim.*, **10**, 187 (1937)
(*11*) Lenhoff, H.M., Nicholas, D.J.D., and Kaplan, N.O., *J. Biol. Chem.*, **220**, 983 (1956)

The Journal of Biochemistry, Vol. 45, No. 10, 1958

NUCLEOTIDES IN ASPERGILLUS ORYZAE*

By KAZUO OKUNUKI, KOZO IWASA, FUMIO IMAMOTO,
AND TADAYOSHI HIGASHIYAMA

*(From the Department of Biology, Faculty of Science,
University of Osaka, Osaka)*

(Received for publication, October 14, 1957)

Recently, the gradient elution of anion exchange chromatography has been established by Potter *et al.* (*1*) in order to separate nucleotides. The method has been applied to the studies of free nucleotides in the biological materials; liver (*1*), mitochondria (*2*), tumor tissues (*3*, *4*), and yeast (*5*). The present paper will participate in a application of the method to separation of the acid-soluble nucleotides of fungal cells.

EXPERIMENTAL AND RESULT

Mycelial mats of *Aspergillus oryzae* grown on a synthetic medium** for 40 hours at 30° were floated on water and wiped with blotting paper. After weighed, the mats were chopped down by scissors in an equal weight of *N* perchloric acid and squeezed for the extraction of nucleotides. The acid extraction was repeated more than two times with 0.5 N perchloric acid. The extract were mixed and adjusted to pH 6.8 with NaOH, and and excess of *M* $BaCl_2$ and then five volumes of 99 per cent ethanol were added. These preparations were carried out at 0±5°. After centrifuged, the precipitated barium salt was washed with 99 per cent ethanol and then with ethyl ether, and dried in a desiccator over calcium chloride. The barium salt was dissolved in 0.5 *N* HCl, and the insoluble matter was removed. The solution was then passed through a Dowex 50 column (Na-form) in order to change the barium salt into sodium salt. The passed solution was applied to a column of Dowex I, X 8 (formate form), 15 cm. in height, 1 cm. in diameter. The gradient elution techniques and the analytical procedures employed were the same as those described by Potter *et al.* (*1*). The chromatography was carried out at first with use of formic acid system in a 250 ml. mixing volume, and then the rechromatography was done using ammonium formate

---

* This work was supported in part by the Grant in Aid for Fundamental Science Research.

** 15 g. of sucrose, 5 g. of $(NH_4)NO_3$, 5 g. of KH_2PO_4, 1 g. of $MgSO_4 \cdot 7H_2O$, trace of $FeCl_3$ and 1000 ml. of tap water.

system in a 500 ml. mixing volume. The optical densities of the successive fractions measured at 260 mμ and the ratio of E_{275} to E_{260} was plotted in Fig. 1. The peak A and B were taken for rechromatography. The base of each nucleotides was distinctly identified by the characterisitics of the absorption spectra from 20 mμ to 300 mμ in the acidic and alkaline conditions. Based upon the elution patterns of the nucleotides (*1*) and and the estimation of molar ratio of acid-labile phosphorus to the nucleic base calculated from molar extinction coefficient, the following nucleotides were found to exist in fungal cell; CMP*, AMP, UMP, CDP, ADP, CTP, ATP, GTP, and UTP, as shown in Fig. 1. GMP, GDP. and DPN, though such nucleotides might hava been thought to exist, could not be detected.

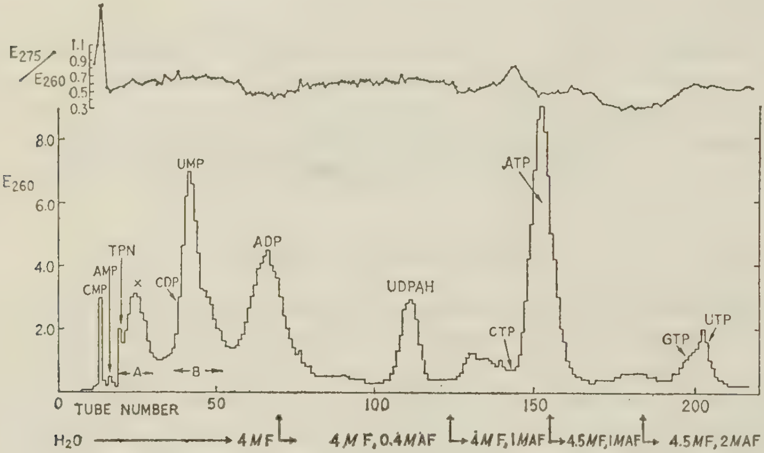

FIG. 1. Chromatography of the acid-soluble fraction of *Aspergillus oryzae* by the formic acid system. Each fraction volume is about 5 ml. 4 *M* F (foamic acid), 0.4 *M* AF (ammonium formate), and so on, indicate the constituent of reservoir solution. The acid-soluble fraction used was prepared from *ca.* 200 g. of mycelial mats. See the text for other details.

Among these nucleotides identified, ATP and ADP were found in large amounts and the others in small amounts, respectively. The result shows that constituents of the nucleotides in fungal cells is analogous to those in yeast cells and animal tissues. The peak A was separated into two parts by the rechromatography; TPN and another unknown component, X, as shown in Fig. 2. TPN could be characterized by the elution pattern and by the shift of its spectrum in the presence of cyanide. The X would appear to be a uridine compound. The peak of fraction-number 105~115 was identified with UDP-acetyl hexosamine (probably glucosamine) by the following analyses; detection of acetylhexosamine (*6*), estimation of acidlabile phosphorus and negative findings in anthrone test (for hexose). The amount of UDPAH was notably varied with the biological condition, and for instance, one of the preparations contained

---

* Following abbreviations were used: MP, monophosphate; DP, diphosphate; TP, triphosphate; A, adenosine; G, guanosine; C, cytidine; U, uridine; TPN, triphospho pyridine nucleotide; UDPAH, UDP-acetyl hexosamine

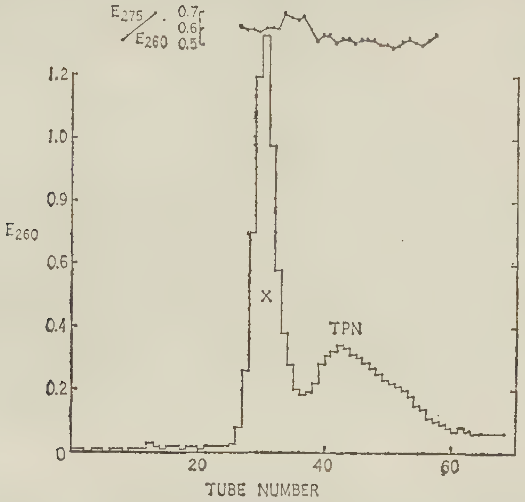

Fig. 2. Rechromatography on the peak A by using the ammonium formate system. 1 *M* of ammonium formate was used in reservoir solution. Other details are the same as Fig. 1.

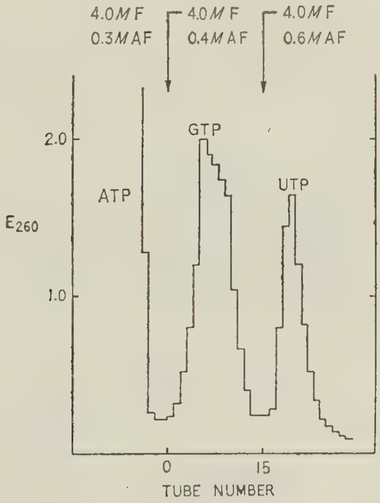

Fig. 3. Hand column chromatography for separation of GTP and UTP. Each fraction volume is about 5 ml.

UDPAH one third as much as the total nucleotides. After all, this fact a large amount of UDPAH is contained in the normal fungal cells is in contrast with the report of Park (*7*) that normal *Staphylococcus aureus* cells contain it in a little amount and the penicillin-treated cells more than 50 folds.

GTP and UTP could be completely separated by a hand column chromatography, as shown in Fig. 3 but not by the gradient elution chromatography.

SUMMARY

By an application of gradient elution system of anion exchange chromatography, acid-soluble nucleotides of *Aspergillus* oryzae were examined.

The nucleotides which have been universally found in yeast cells and animal tissues, exist also in the fungal cells; CMP, AMP, UMP, CDP, ADP, CTP, ATP, UTP, GTP, TPN, and UDP-acetyl hexosamine. And the cells contains another unknown nucleotide having uridine base. ATP, ADP, and UDPAH exist in relatively large amounts compared with the others.

The helpfull aids of Dr. Y. Takagi, Medical School of Osaka University, and of Dr. Y. Imanaga, Nara Woman's University, on the assay of acetyl hexosamin, are gratefully acknowledged.

REFERENCES

(*1*) Hurlbert, R.B., Schmitz, H., Brumm, A.F., and Potter, V.R., *J. Biol. Chem.*, **209**, 23 (1954)

(*2*) Brumm, A.F., Potter, V.R., and Siekevitz, P., *J. Biol. Chem.*, **220**, 713 (1956)

(*3*) Schmitz, H., Potter, V.R., Hurlbert, R.B., and White, D.M., *Cancer Research*, **14**, 66 (1954)

(*4*) Schmitz, H., *Biochem. Biophy. Acta*, **14**, 160 (1954)

(*5*) Schmitz, H., *Biochem. Z.*, **325**, 555 (1954)

(*6*) Morgan, W.G.J., and Elson, L.A., *Biochem. J.*, **28**, 988 (1934)

(*7*) Park, J.T. Phosphorus Metabolism; edited by Mc Elroy, W.D., and Glass, B., John Hopkins press. Baltimore, Vol. **I** p. 93 (1951)

The Journal of Biochemistry, Vol. 45, No. 10, 1958

SPECIFIC ACTIVITY OF LIVER DEOXYRIBONUCLEIC ACID LABELLED WITH P^{32}*

BY MINORU AMANO**, HIROJI K. KIHARA*** AND
ATUHIRO SIBATANI

(*From the Cytochemistry Laboratory, Yamaguchi Medical School, Ube*)

(Received for publication, April 30, 1958)

The contamination of P^{32} in deoxyribouncleic acid (DNA) offers a serious problem in tracer experiments especilly with material of low DNA-synthesising activity. In this paper we want to show that the data on DNA of adult rat livers as presented by Stevens *et al.* (*1*). may be too high and can not be used as the basis of their calculation to correlate the rate of DNA synthesis with mitotic rate of this organ.

EXPERIMENTAL

Animals—Adult rats of unspecified strains weighing 190–210 g. received a single intreperitoneal injection of P^{32}-orthophosphate solution (100–200 μc.) 24 hours before sacrifice. Individual livers were processed separately.

Isolation of Nucleic Acids—Method I: This method was used in our previous experiments (*2–4*). Following extraction with cold trichloroacetic acid (TCA) and the delipidation by the routine procedure of the homogenised tissues, the nucleic acids were extracted 3 times with decreasing volumes of 10 per cent NaCl solution at 100° far 4 hours each, precipitated with ethanol, dissolved in 10 per cent NaCl by heating at 85° for 15 minutes, reprecipitated with ethanol, taken up in water, and precipitated with 0.1 volume of *N* HCl. DNA and ribonucleic acid (RNA) were separated accordind to Barnum *et al.* (*5*).

Method II: This is a modifications of Method I for an extensive purification, especially of DNA. Tissue homogenate was extracted with cold TCA, and the residue was washed with 80 per cent ethanol, treated overnight with ethanol saturated with sodium acetate to eliminate the trace of acidity (*6*), delipidated with hot ethanol-ether (3 : 1) as usual and extracted with 10 per cent NaCl in 0.05 *M* veronal-acetate buffer (*7*) of pH 7.0 at 100° 4 times for 1 hour each and then twice for 4 hours each. The combined extracts were treated as in Method I. The reprecipitated crude nucleic acids were

---

* Aided in part by Grants for Fundamental Scientific Research of the Ministry of Education. p^{32} was received from the Radiochemical Centre, Amersham, England, on allocation of JRA.

** Present address: Department of Anatomy, McGill University, Montreal, Canada.

*** Present address: Department of Bacteriology, College of Agriculture, University of Wisconsin, Madison, Wisconsin, U.S.A.

taken up in physiological saline and treated with 0.1 volume of 5 per cent dodecyl sulphate (purified from Emal, product of Kaoo Sekkn, Tokyo) solution in 45 per cent ethanol at 30–40° for 2–3 hours with occasional stirring. The concentration of NaCl was then raised up to 1 *M*, and after standing at room temperature for 1 hour, the mixture was centrifuged; the supernatant was treated with 1 volume of acetone and and 2 volumes of ethanol. The nucleic acid precipitate was taken up in a small volume of water and further deproteinised by shaking repeatedly with small volumes of chloroform. Nucleic acids were precipitated from the aqueous layer with 1 volume of ethanol containing 0.2 *N* HCl. After separation of RNA by alkaline hydrolysis (0.1 *N* NaOH at 80° for 10 minutes) and reacidification as in Method I, the preceipitate of DNA was dissolved in 0.1 *N* NaOH containing alkalihydrolysed non-radioactive yeast RNA in 5–10 times the amount of RNA of the sample, heated at 80° for 20 minutes, and reprecipitated with 0.2 volume of cold *N* HCl after chilling. DNA was then dissolved repeatedly in 0.1 *N* NaOH without addition of carrier RNA, twice with heating as above, and twice without heating, and reprecipitated each time with cold *N* HCl as above. It was finally digested with 5 per cent TCA as in other methods.

Method III: In this method DNA was first extracted in a highly polymerised form by a small scale modification of the method of Kay *et al.* (*8*). Nuclei isolated with citric acid and suspended in 0.14 *M* NaCl (*9*) were treated with dodecyl sulphate as in Method II. A fibrous ethanol-precipitate of DNA, after thorough washing with acetone and ethanol, was further purified as in Method II to give DNA free from contaminating RNA. No carrier RNA was added at the last stage when we used this method in a previous work (*4*)*.

Total Acid-soluble P and the Determination of Radioactivity—These were the same as in a previous report (*4*). Results are expressed in relative specific activity and in apparent relative uptake (*3*).

RESULTS AND DISCUSSION

The specific activity of DNA of the liver of normal adult rats as obtained with Method I was quite low but certainly not negligible. As shown in Table I, Methods II and III, involving more extensive purification of DNA, could reduced the specific activity of DNA down to about half that obtained with Method I. It is probable that this decrease was due to the elimination of contamination and the values obtained by Methods II and III represent, at least in part, the residual net DNA synthesis in adult rat liver.

The comparison of our values with those reported by Stevens *et al.* (*1*) may be feasible, because the experimental conditions were quite comparable. Table II shows that our values obtained with Method I agree very well with those of the Canadian workers who used the simpler

---

* After completion of this work, we have further improved the method, the final procedure being used in several recent works (*10–12*). This is essentially in accordance with the method of Sibatani (*13*) for C^{14}-work, but extraction of nucleic acids with hot dodecyl sulphate was conducted with tissue residue extracted with cold TCA as in Method II, and was prolonged for 30 minutes and then for another 30 minutes in *M* NaCl. Separatian of RNA and DNA was as in Method I.

Schmidt-Thannauser produre. It is thus highly likely that the rate of DNA synthesis as given by Stevens *et al.* for adult rat liver was at least twice as high as the true value. This is an additional evidence suggesting that, contrary to their conclusion, DNA is not equilibrated with the preursor pool throughout the course of mitotic cycle (*4*, *14*, *15*).

TABLE I

Specific Activities of P^{32}-Labelled Nucleic Acids in the Liver of Adult Rats

Each rat received 100–200 μc. of P^{32}-orthophosphate 24 hours before sacrifice. In method III only DNA was separated and the ratio RNA-P/DNA-P was calculated using the value of RNA-P obtained by Method II with the same material.

Rat No.	Method	Relative specific activity			
		Acid-sol. P	DNA-P	RNA-P	$\frac{\text{RNA-P}}{\text{DNA-P}}$
1	I	3.94	0.134	1.15	8.6
2	I	3.27	0.072	0.968	13
3	II	4.06	0.046	1.62	35
	III		0.074		22
4	II	3.30	0.055	1.33	24
	III		0.048		28
5	II	5.35	0.046	1.12	24
	III		0.044		25

TABLE II

Specific Activities of DNA in Rat Liver as Obtained by Different Methods

Method	Relative specific activity	Apparent relative uptake
I	0.103	2.8
II	0.049	1.2
III	0.055	1.4
Schmidt-Thannhauser (*1*)	0.11	3.0

The specific activity ratios RNA/DNA of normal rat liver as obtained with Methods II and III were much higher than the ones obtained with Method I (Table I), approaching and sometimes exceeding the frequently cited value of 33 (obtained 2 hours after P^{32} administration) as reported by Hammarsten and Heavesy (*16*). Those values are of the same order of magnitude as the ratios reported in a previous paper (*13*) under similar experimental conditions but using C^{14}-formate and [2-C^{14}] glycine as the precursor, and range themselves among the highest of RNA/DNA ratios obtained so far in normal liver with P^{32} as the tracer (*17*).

SUMMARY

The radioactive contamination of deoxyribonucleic acid (DNA) in the normal liver of adult rats receiving P^{32} may be eliminated only by an extensive purification. It is suggested that the rate of DNA synthesis as estimated by the P^{32} incorporation does not exceed the mitotic rate in this organ. This is an additional evidence for the matabolic stability of DNA.

REFERENCES

(*1*) Stevens, C.E., Daoust, R., and Leblond, C.P., *J. Biol. Chem.*, **202**, 177 (1953)
(*2*) Fujisawa, Y., and Sibatani, A., *Experientia*, **10**, 178 (1954)
(*3*) Kihara, H.K., Amano, M., Ikemoto, H., and Sibatani, A., *Biochim. Biophys. Acta*, **17**, 143 (1955)
(*4*) Kihara, H.K., Amano, M., and Sibatani, A., *Biochim. Biophys. Acta*, **21**, 489 (1956)
(*5*) Barnum, C.P., Nash, C.W., Jennings, E., Nygaard, O., and Vermund, H., *Arch. Biochem.*, **25**, 376 (1950)
(*6*) Marco, A.M., and Butler, G.C., *J. Biol. Chem.*, **190**, 165 (1951)
(*7*) Roth, J.S., *J. Biol. Chem.*, **203**, 181 (1954)
(*8*) Kay, E.R.M., Simmons, N.S., and Dounce, A.L., *J. Am. Chem. Soc.*, **74**, 1724 (1953)
(*9*) Mirsky, A.E., and Pollister, A.W., *J. Gen. Physiol.*, **30**, 117 (1946)
(*10*) Osawa, S., Takata, K., and Hotta, Y., *Biochim. Biophys. Acta*, **25**, 656 (1957)
(*11*) Naora, H., and Naora, H., *Gann*, **48**, 255 (1957)
(*12*) Sekiguchi, M., and Sibatani, A., *Biochim. Biophys. Acta*, **28**, 455 (1958)
(*13*) Sibatani, A., *Biochim. Biophys. Acta*, **25**, 592 (1957)
(*14*) Daoust, *R*., Leblond, C.P., Nedler, N.J., and Enesco, M., *J. Biol. Chem.*, **221**, 727 (1956)
(*15*) Swick, R.W., Koch, A.L., and Handa, D.T., *Arch. Biochem. Biophys.*, **63**, 226 (1956)
(*16*) Hammarsten, E., and Hevesy, F., *Acta Physiol. Scand.*, **11**, 335 (1946)
(*17*) Brown, G.B., and Roll, P.M., in "The Nucleic Acids", ed. by Chargaff, E., and Davidson, J.N., Academic Press, New York, Vol. **2**, p. 341 (1955)

The Journal of Biochemistry, Vol. 45, No. 10, 1958

STUDIES ON THE RIBONUCLEOPROTEIN PARTICLES

III. SOME PHYSICOCHEMICAL CHARACTERISTICS AND THE DEGRADATION WITH RIBONUCLEASE ANC TRYPSIN OF THE MICROSOMAL RIBONUCLEOPROTEIN PARTICLES

By YUTAKA TASHIRO

(*From the Department of Physiology, Faculty of Medicine
Kyoto University, Kyoto*)

(Received for publication, May 16, 1958)

In the earlier studies, the authors have described the isolation and purification of the ribonucleoprotein particles from the rat liver microsomes, and they reported the preliminary studies on the degradation of these particles with ribonuclease and with trypsin (*1*, *2*), and also investigated the ribonuclease activity of these particles, (*2*, *4*, *5*), in addition to an electronmicroscopical study on the internal structure of these particles (*3*).

The present work attempts to study some physicochemical properties and enzymatic degradation of these particles in more detail, and to estimate the structure of the these particles.

EXPERIMENTALS

Isolation and Purification of the Ribonucleoprotein Particles—Ribonucleoprotein particles were isolated from the rat liver microsomes with sodium deoxycholylate and then purified by the repeated low and high centrifugations as described previously (*6*, *1*, *2*). All the procedures were performed in the cold (0–5°).

Degradation with Ribonuclease or with Trypsin—Ribonucleoprotein was incubated without adding enzyme, with ribonuclease, or with trypsin. After a definite interval, an aliquot of ice cold *N* perchloric acid was added and the amount of nucleic acid and protein in the precipitate was determined by the Schmidt Thannhauser's method slightly modified by Littlefield *et al.* (*6*). Crystalline ribonuclease was obtained from the Wako Pure Chem. Ind. Osaka and trypsin, twice crystalized and salt free, was obtained from the Washidgton Biochem. Corp. They were previously dissolved in the same buffer.

RESULTS

1. Effect of pH upon the Ribonucleoprotein—To each 0.5 ml. of the ribonucleoprotein solution (about 0.5 per cent) was added 4 ml. of the Miller-Golder's buffer (*7*) of various pH value and of ionic strength

0.1 at 0°, and the optical density at 520 mμ was read by a shimazu spectrophotometer. As shown in Fig. 1, the turbidity of the solution increased abruptly at pH 5~6, and reached a maximum at pH 3.2-3.5.

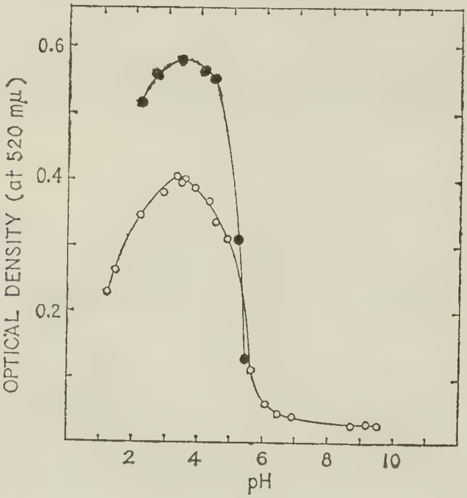

Fig. 1. Effect of pH upon the turbidity of the ribonucleoprotein solution.

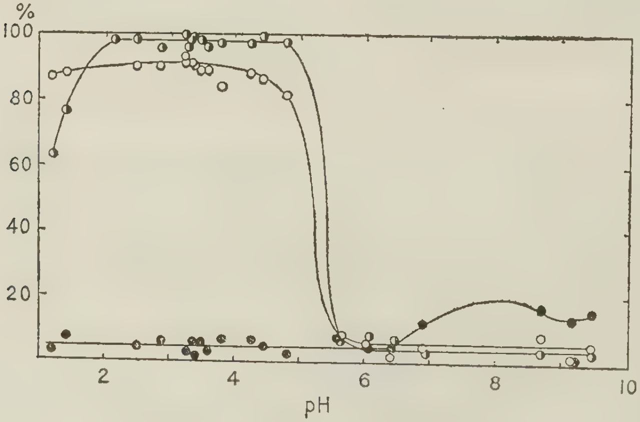

Fig. 2. Effect of pH upon the macromolecular composition of the ribonucleoprotein. (◑), per cent of protein in the sediment; (○), per cent of ribonucleic acid in the sediment; (●), per cent of acid soluble ribonucleic acid in the supernatant.

This result suggests that the isoelectric point of the ribonucleoprotein is more acidic than previously reported (*8*), and that acidic micromolecules such as nucleic acid probably exists on the surface of the particles.

After the turibidimetry, each solution was centrifuged at 3,000 r.p.m. for 20 minutes in the cold to determine the nucleic acid and protein content in both supernatant and sediment, respectively. In acidic region of pH 2 to 4, most nucleic acid (○) and protein (◑) were found in the sediment, while in neutral or in alkaline region of pH 6~10, they were almost exclusively found in the supernatant (Fig. 2)

Therefore in seems probable that the ribonucleoprotein exists stably from pH 2 to 9, and does not dissociate inso component macromolecules. The increase of acid soluble nucleic acid (●) at pH 7 to 8, is due to the ribonuclease activity of the particles in themselves as reported later (*5*). The remarkable release of protein from the sediment at pH 1~2 suggests that dissociation of the nucleoprotein to nucleic acid and protein occurred in such acidic region.

This estimation has also been proved by the electrophoretical study in which the ribonucleoprotein migrates as a single peak at pH 6–9, whereas in more alkaline solution of pH 12.0, it was dissociated into two peaks*.

2. *Degradation of the Ribonucleoprotein Particles with Pancreatic Ribounclease*—The rate at which ribonucleic acid is degraded with ribonuclease depends not only upon various environmental conditions, but also upon the physical state of the substrate or enzyme.

For example, when native tobacco mosaic virus** is incubated with pancreatic ribonuclease (○) at pH 7.0 and at 37°, no nucleic acid was degraded at all as shown in Fig. 3.

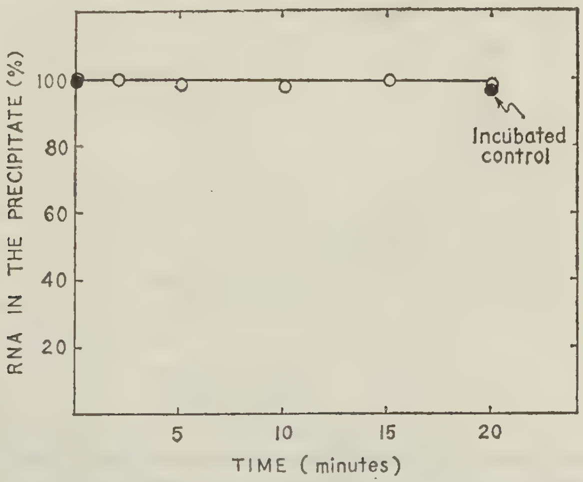

FIG. 3. Action of pancreatic RNase upon tobacco mosaic virus.

---

* Y. Tashiro, unpublished.

** Tobacco mosaic virus was generously suppylied by Dr. Z. Hidaka of Hatano Tobacco Experiment Station, Kanagawa.

The similar inactivity of ribonuclease was also observed when the coacervate of nucleic acid with protamine was incubated with the enzyme (Fig. 4). It appears from these results that, when nucleic acid is covered by protein or is in salt-like linkage with basic protein, nucleic acid is not degraded at all or is difficult to be digested.

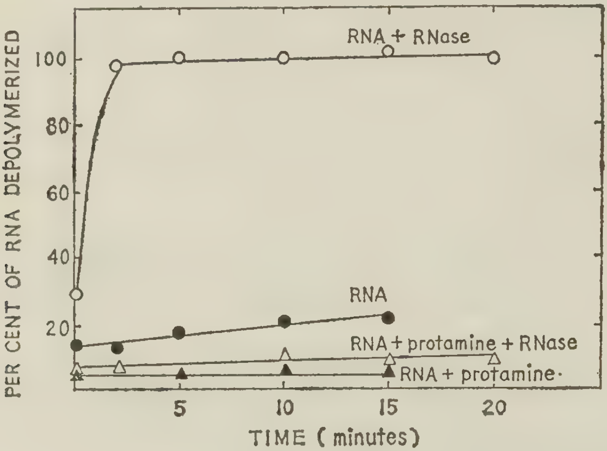

Fig. 4. Action of pancreatic RNase upon free yeast RNA (○) and RNA-protamine coacervate (△).

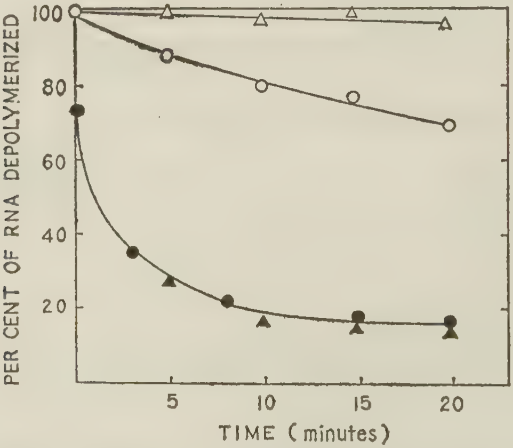

Fig. 5. Effect of ribonuclease upon the ribouncleic acid of the microsomes (▲) and the ribonucleoprotein particles (●). As a control, the microsomes (△) and ribonucleoprotein (○) were incubated without ribonuclease.

Fig. 5. illustrates that ribonucleic acid of the isolated ribonucleoprotein (●) or of the microsomes (▲) were easily digested with ribonuclease, suggesting that the physical state of ribonucleic acid in these particles is quite different from that of the tobacco mosaic virus or of the ribonucleic acid-protamine coacervate. This is one of the reasons why the author has estimated that a considerable amaunt of ribonucleic acid exists on the surface of the particles.

It is also to be noted that about 15 per cent ribonucleic acid was not digested after prolonged incubation with ribonuclease. Novikoff *et al.* (*10*) have described a similar observation and estimated that smaller polynucleotides rich in purine bases remained after the digestion. This is to be examined later.

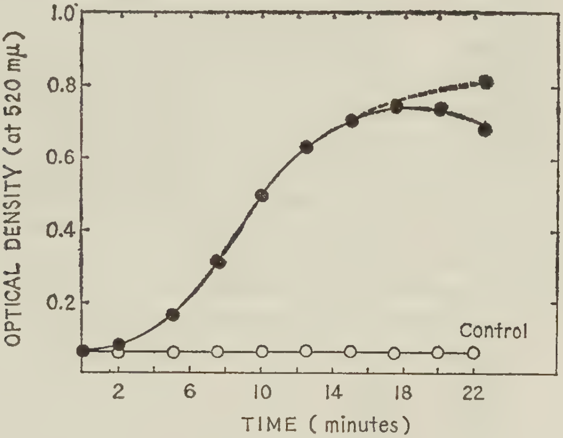

Fig. 6. Turbidity increase of the ribonucleoprotein solution by the removal of nucleic acid from the particles.

3. *Turbidity Increase of the Ribonucleoprotein Solution by the Incubation with Ribonuclease*—As shown in Fig. 6, turbidity of the ribonucleoprotein solution increased remarkably when incubated with ribonuclease at 37°, and reached a maximum after 16∼18 minutes. The drop in optical density after about 20 minutes is probably due to the flocculation induced by the mutual aggregation of the particles, because, such a drop in optical density was not observed when the solution was incubated with continuous stirring.

Comparing Fig. 6 with Fig. 5, it is estimated that the stability of the particles denpends much upon the negative charge of the ribonucleic acid on the surface of the particles, since acidification of the solution by the incubation with ribonuclease was too slight (0.1∼0.2 by ph unit) to attribute the agregation of the particles to acidification of the solution.

4. *Degradation of the Ribonucleoprotein Particles with Trypsin* (Fig. 7)—When 1.0 ml. of 0.5 per cent ribonucleoprotein solution was incubated with an aliquot of 0.01 per cent trypsin at 37°, 30–50 per cent of protein

(●) and ribonucleic acid (◐) were digested within 20 minutes.

In Fig. 7, (○) and (◑) represent respectively the decrease of protein and nucleic acid of the incubated control. It is not yet certain whether

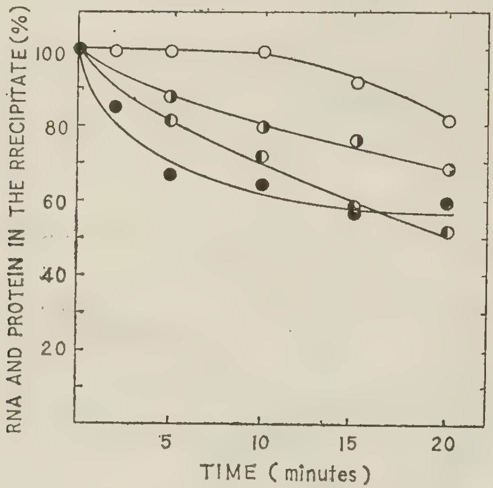

Fig. 7. Degradation of the ribonucleoprotein with trypsin.

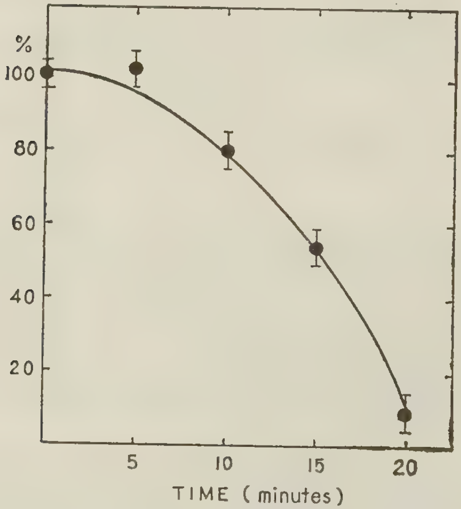

Fig. 8. Decrease of specific radioactivity of labelled microsomal ribonucleoprotein (C^{14}-leucine) by incubation with trypsin.

the accelerated degradation of ribonucleic acid by trypsin is due to the activation of the ribonuclease activity of the particles in themselves, or to the very small amount of ribonuclease contaminating in trypsin.

5. Degradation of the C^{14}-Leucine Labeled Ribonucleoprotein Particles with Trypsin—On the basis of the experiments especially with electrophoresis (*1*, *4*), the postulation has been made that the ribonucleoprotein particles are composed of two parts, that is, the labile and stable component, and usually, the later is covered by the former.

In order to compare the metabolical activity of the both, the ribonucleoprotein was extracted from the rats which had received C^{14}-leucine 5 minutes before decaptation, and labeled nucleoprotein was incubated with trypsin at 37°. After a definite interval, an aliquot of ice cold *N* perchloric acid was added and the radioactivity of protein in the precipitate was measured by a flow counter.

As shown in Fig. 8, radioactivity per unit weight of protein remarkably decreased by the incubation with trypsin.

6. Ultracentrifugal Studies on the Degradation of the Ribonucleprotein Particles—1 ml. of about 0.5 per cent ribonucleoprotein solution was incubated at 37° with an aliquat of 0.01 per cent ribonuclease or trypsin solution previously dissolved in the Miller-Golder's buffer of pH 7.0 or with 1 ml. of the buffer as a control. After a definite interval, they were rapidly cooled again, 7 ml. of the ice cold buffer solution was added, and were contrifuged at once at $105,000\times g$. for 120 minutes. The supernate and pellet was separated by decantation and the nucleic acid and protein content of both fractions were determined by Schmidt-Thannhauser's method.

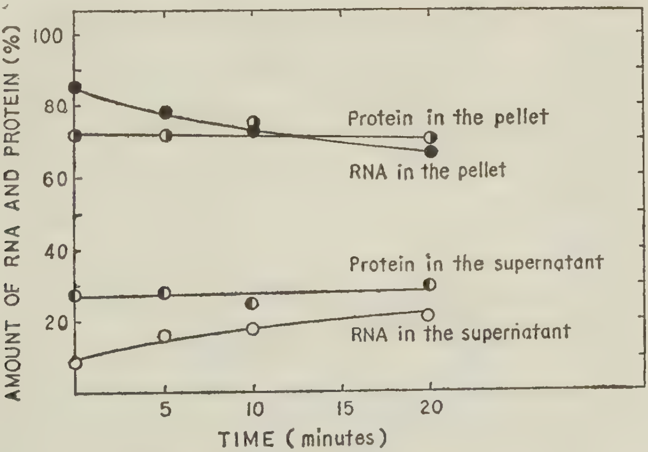

Fig. 9. Ultracentrifugal study on the ribonucleoprotein incubated without adding enzyme.

Fig. 9, Fig. 10 and Fig. 11 show the ribonucleoprotein incubated without adding enzyme, with ribonuclease and with trypsin respectively.

In Fig. 9, the gradual decrease of nucleic acid in the pellet (●) is due to the increase of both acid isoluble and soluble ribonucleic acid in the supernatant. In spite of the decrease of nucleic acid, protein in the pellet (◑) was kept at a constant level. Therefore it seems probable that the molecular weight of the particles decreased not so remarkably by such release of nucleic acid.

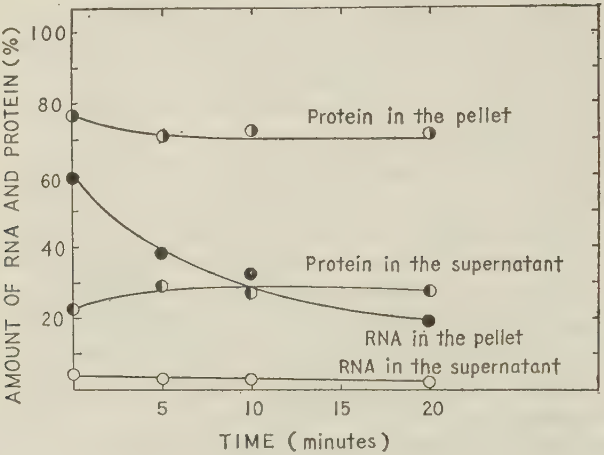

Fig. 10. Ultracentrifugal study on the ribonucleoprotein incubated with RNase.

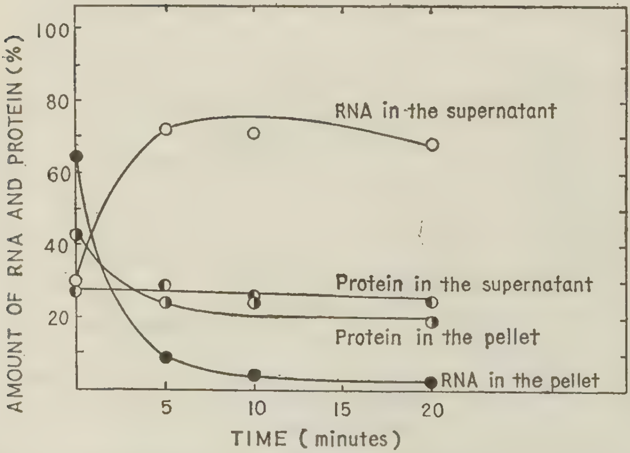

Fig. 11. Ultracentrifugal study on the ribonucleoprotein incubated with trypsin.

It is the most interesting finding in the Fig. 10 that almost all the acid insoluble ribonucleic acid (●) was found in the pellet. Therefore, the ribonucleic acid which was resistant to ribonuclease is probably not a smaller polynucleotide as suggested by Novikoff (*10*) but a nucleoprotein complex with considerable molecular weight. The base composition and other physicochemical properties of the ribonuclease resistant core are to be further examined by the author.

When the ribonucleoprotein was treated with trypsin, protein (◐) and especially nucleic acid (●) in the pellet decreased remarkabley as shown in Fig. 11, while, the acid insoluble nucleic acid in the supernatant (○) increased remarkable at first and then gradually decreased probably by the degradation with ribonuclease. Therefore trpsin probably destructed the nucleoprotein particles into smaller fragment which were now not sedimentable by centrifugation at 105,000×*g* for 120 minutes.

7. A Preliminary Experiment on the Reconstruction of the Ribonucleoprotein Particles—The ribonucleoprotein was incubated with ribounclease for about 30 minutes to remove nucleic acid and centrifuged in the cold. The pellet was washed three times with ice cold Miller-Golder's buffer or pH 7.0 and of ionic strength 0.1. The protein obtained in such a manner was suspended in the cold 0.1 *N* NaCl solution. To each 1.0 ml. of this solution, 4.0 ml. of the Miller-Golder's buffer with various pH value was added and the optical density at at 520 mμ was read as usual.

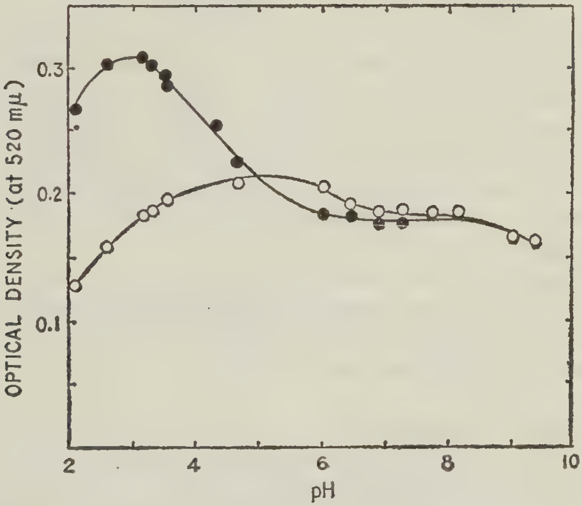

FIG. 12. Turbidimetric analysis on the protein of the ribonucleotein (○) and the change of turbidity induced by the addition of yeast ribonucleic acid to the protein (●).

As shown in Fig. 12, the protein was hardly soluble over pH 2 to 9 avd the maximum turbidity was about at pH 5~6.

After turbidimetry, to 2.5 ml. of the solution was added 0.5 ml. of yeast ribonucleic acid which corresponds just to the ribonucleic acid removed previously by ribonuclease and to another 2.5 ml. of the solution was added 0.5 ml. of the buffer as a control.

In the acidic region, turbidity of the solution (●) remarkably increased, while in neutral region (pH 6–8), the turbidity decreased slightly when compared with the control.

This finding can reasonably be interpreted by the binding of yeast nucleic acid with the protein, because complex formation between the protein and yeast nucleic acid should result in the aggregation of the particles in acidic region and result in the redispersion by the mutual repulsion of the particles in neutral region. More quantitative experiment is now under investigation by the author.

DISCUSSION

According to the from in which nucleic acid exists, three type of nucleoproteins can be distinguished. (1) The covered (masked) type, in which nucleic acid is covered by protein as in the case of bacteriophage (*11*), tobacco mosaic virus (*12*) and turnip yellow mosaic virus (*13*) in their resting state. This type is biologically inactive in usual, and is probably a protecting form for nucleic acid. (2) The covering (masking) type, in which nucleic acid covers a protein core. (3) The intermediate (mixed) type, in which polynucleotide chains are associated directly with polypeptide chains, as suggested by Elson and Chargaff (*14*).

For the arrangement of ribonucleic acid in the ribonucleoprotein particles, it was suggested in the previous paper (*1–4*) that a considerable part of ribonucleic acid should exist on the surface of the particles probably as a intermediate type (labile nucleoprotein) and that the remainder might exist in the core of the particle as a covered type (stable nucleoprotein).

These assumptions also receive support from the present experiments. By the incubation with ribonuclease or bp autodegradation, the nucleic acid on the surface seems to be very unstable and easily depolymerized, while the nucleic acid in the core seem to be resistant to ribonuclease and stable.

At the same time the surface of the particles seems to be more actively engaging in the protein synthesis than the core. This assumption receives support from observation made by Littlefield *et al.* (*6*) showing that only a very small fraction of the total amino acids in the particles is turning over rapidly.

SUMMARY

Some physicochemical characteristics of the ribonucleprotein particles isolated from the rat liver microsomes with sodium deoxycholate were examined, and the particles were digested with ribonuclease and with

trypsin to estimate the internal structure of the particles.

1. The isoelectric point of the particles was estimated to be pH 3.2~3.6 by turbidimetry. When pH of solution was kept between pH 2~9, these particles were stable and did not dissociate into the component macromolecules. Whereas below pH 2, considerable protein was released from the particles.

2. About 80–85 per cent of ribonucleic acid of the particles was easily removed by the incubation at 37° for 20 minutes with pancreatic ribonuclease. The removal of nucleic acid resulted in the turbidity increase of the solution probably due to the mutual aggregation of the particles.

3. Trypsin digested about 30–50 per cent protein of the particles at 37° within 20 minutes. When the ribonucleoprotein particles previously labeled with C^{14}-leucine was digested with trypsin, the ratioactivity per unit weight of protein decresed remarkable with time.

4. Ultracentrifugal studies demonstrated that the core nucleic acid, which was resistant the ribonuclease, was sedimented by centrifugation at 105,000×*g*. for 120 minutes. Therefore the core nucleic acid is probably not a small polynucleotide as suggested by Novikoff, but a macromolecular one, and is either covered by protein as in the case of tobacco mosaic virus or is firmly combined with basic protein as in the case of nucleic acidprotamin complex.

Trypsin destructed the particles into smaller fragments, which were not sedimented by the same centrifugal forces.

5. The protein part of the ribonucleoprotein obtained by the incubation with ribonuclease was hardly soluble to Miller-Golder's buffer of pH 2~9 and its maximum turbidity was at pH 5~6. This protein combines easily with yeast ribonucleic acid, and the combination of the both resulted in the turbidity increase in acidic region (pH 2~5) and the turbidity decrease in neutral region (pH 6~8).

6. From these findings there appears no doubt that a considerable part of ribonucleic acid exist on the surface of the particles to give them the negative charge, which is probably the most important factor for the stabillty of the particles. Further it was estimated that there existed the stable nucleoprotein in the core of the particles, of which metabolic activity was far less active than the surface.

The author wishes to express his thanks to Prof. K. Sasagawa and to Prof. A. Inouye for their encouragement.

REFERENCES

(*1*) Tashiro, Y., Sato, A., Takai, K., Hirakawa, K., and Hirano, S., *J. Jap. Biochem, soc.* **29**, 169 (1957)

(*2*) Tashiro, Y., and Sato, A., *Kagaku*, **27**, 367 (1957); *J. Jap. Biochem, Soc.* **29**, 615 (1957)

(*3*) Tashiro, Y., Sato, A., and Furuta, Y., *Cytologia*, **22**, 136 (1957)
(*4*) Tashiro, Y., Ogura, M., Sata, A., Shinagawa, Y., Imai, Y., Hirakawa, K., and Hirano, S., *Proc. Intern. Symp. Enz. Chem.*, Tokyo and Kyoto, p. 436 (1957)
(5) Tashiro, Y., *J. Biochem.*, in press (1958)
(*6*) Littlefield. J.W., Keller., E.B., Gross, J., and Zamecnik, P.C., *J. Biol.* Chem., **217**, 111 (1955)
(*7*) Miller, G.L., and Golder, R.H., *Arch. Biochem.*, **29**, 420 (1950)
(*8*) Magasanik, B., in "The Nucleic Acids", edited by Chargaff and Davidsoh, **1**, 373 (1955)
(*9*) Tashiro, Y., *Symp. Soc. Cell. Chem.*, **7**, in press (1958)
(*10*) Novikoff, A.B., Ryan, J., and Podber, E., *J. Histochem. Cytochem.* **1**, 27 (1953)
(*11*) Hershey A.D., and Chase, M., *J. Gen. Physiol.*, **36**, 39 (1952)
(*12*) Hart, R.G., *Proc. Natl. Acad. Sci. U.S.A.*, **41**, 261 (1955)
(*13*) Markham, R. and Smith, J.D., *Parasitol.*, **39**, 330 (1949)
(*14*) Elson, D., and Chargaff, E., *Biochim. Biophys. Acta*, **17**, 367 (1955)

The Journal of Biochemistry, Vol. 45, No. 10, 1958

STUDIES ON CYTOCHROME C

II. FURTHER STUDY ON PROTFINASE DIGESTION OF BAKER'S YEAST CYTOCHROME C

By MITSUHIRO NOZAKI, HIROSHI MIZUSHIMA, TAKEKAZU HORIO
AND KAZUO OKUNUKI

*(From the Departmemt of Biology, Faculty of Science,
University of Osaka, Osaka)*

(Received for publication, February 7, 1958)

In the previous paper (*1*), it was confirmed that our method for preparation of crystalline cytochrome c is suitable (*2*), even after several critical examinations of the purification procedure. By this procedure we can prepare selectively yeast cytochrome c and experimentally it appears to have the same protein configuration as that existing in the respiring yeast cells, that is the "native" protein configuration. However by chromatography on cation exchanger column during the purification procedure, yeast cytochrome c is separated into various fractions and almost all have the same absorption spectrum and can undergo chemically and enzymically oxidation and reduction as well as the native form. Trichloroacetic acid (TCA) rapidly modifies the native yeast cytochrome c even at low temperature and at the neutral pH. This modified yeast cytochrome c appears chromatographically in the same fraction as yeast cytochrome c modified by boiling but can be separated from the native form. These modified cytochrome c's are indistinguishable from the native form by their absorption spectra and by reduction with yeast lactic dehydrogenase (*1*). However, the modified forms are digested by bacterial proteinase more rapidly than the native form (*1*). Though yeast cytochrome c, even in its native state, is digested rapidly by the proteinase, it is much more resistant to digestion in its reduced than in its axidized form. This fact indicates that the form of the heme-protein is changed very much by the simple change of covalence of the iron of its heme-moiety. The stabilily of cytochrome c in oxidized and reduced forms may make it advantageous to study the relations between protein configuration and function.

This paper is concerned with digestion of yeast cytochrome c by trypsin and also with a study of digestion by bacterial proteinase. Under anaerobic condition, native yeast cytochrome c in its reduced form is scarcely digested by bacterial proteinase, while it in its oxidized form is digested rapidly.

MATERIALS AND METHODS

Preparation of Crystalline Yeast Cytochrome c—This was prepared by the same method as in aur previous paper (*1*). The yeast cytochrome c which corresponds to fraction IV on an Amberlite XE-64 chromatogram (*1*) was used as native yeast cytochrome c.

Preparation of Crystalline Bovine Heart Muscle Cytochrome c—Method as described in our previous paper (*3*).

Preparation of Yeast Lactic Dehydrogeaase—Method as described in our previous paper (*4*).

Digestion of Cytochrome c by Trypsin—Lyophilized trypsin powder from Mochida Phamaceutical Ltd., Co., Tokyo, was dissolved in 0.005 M HCl to give 0.12 per cent final concentration and stored in a frozen state. For use, the stock solution was diluted with 0.1 M phosphate buffer of pH 8.0 to give a concentration which would digest casein at the same rate as the solution of bacterial proteinase (*1*). The methods used for digestion and for calculation of the percentage digestion were the same as those for the bacterial proteinase (*1*).

Preparation of Oxidized and Reduced Cytochrome c's—Cytochrome c solution was completely oxidized and reduced by addition of a minimum amount of potassium ferricyanide, and sodium dithionite or sodium ascorbate, respectively, at a neutral pH, and were then dialyzed against 0.02 M Na_2HPO_4 solution overnight in a refrigerator. The phosphate solution was renewed two or three times during the period. It was established that this procedure did not influence yeast cotochrome c, for like the native one it appeared in fraction IV on an Amberlite XE-64 column (*1*).

Digestion of Cytochrome c by Bacterial Proteinase—Proteinase digestion and calculation of the percentage digestion were carried out by the method described in our previous paper (*1*).

Digestion of Yeast Cytochrome c by Proteinaie in Anaerobic Condition—Thunberg tubes were use for this purpose: One ml. of yeast cytochrome c was put in the main chamber and 0.5 ml. of proteinase solution in the side chamber of the tube. They were evacuated and then filled with oxygen-free nitrogen gas. This procedure was repeated three times. Reaction was started by mixing of the contents of the two chambers and stopped by addition of 3.0 ml. of 0.6 M TCA to each tube.

Measurement of Azsorption Spectrum—This was done in a cuvette of one cm. optical path in a Shimidzu photoelectric spectrophotometer, type QB-50. Changes of absorption spectrum under anaerobic conditions were measured using a Thunberg type glass cuvette of one cm. optical path. Anaerobic conditions were obtained as described in the above section.

TCA-Treated and Boiled Preparations of Cytochrome c—These were abtained as described in our previous paper (*1*). These preparations of yeast cytochrome c appeared in fraction I when chromatographed on an XE-64 column (*1*) and will be referred to as "modified" yeast cytochrome c in the following sections.

RESULTS

Comparison of Digestion of Yest Cytochrome c by Bacterial Proteinase and Trypsin—Digestion of oxidized, native yeast cytochrome c by trypsin was compared with that by bacterial proteinase using the proteinase solutions which were previously adjusted to concentrations which would digest casein at the same rate. As shown in Fig. 1, oxidized, native yeast cytochrome c is rapidly digested by trypsin and at almost the same rate as by bacterial proteinase.

Digestion of Yeast and Bovine Cytochrome c's by Trypsin—Oxidized yeast cytochrome c is much more rapidly digested by trypsin than oxidized bovine cytochrome c, as shown in Fig. 2. When modified by TCA or by boiling (*1*), yeast cotochrome c is more susceptible to digestion by trypsin. As is the case in bacterial proteinase digestion (*1*), reduced, native yeast cytochrome c is less susceptible to the digestion than the oxidized form.

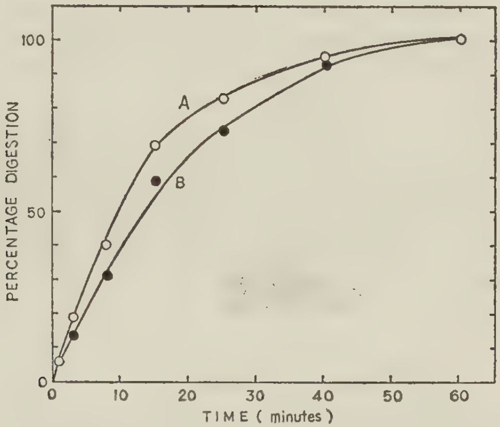

Fig. 1. Comparison of digestions of yeast cytochrome c by bacterial proteinase and trypsin.

Oxidized, native yeast cytochrome c containing 10 per cent of the reduced form, was used as the substrate. The reaction mixture was as follows: Cytochrome c, 0.075 per cedt (calculated assuming that its molecular weight is 13,000); bacterial proteinase, 0.005 per cent or trypsin adjusted to digest casein at the same initial rate as bacterial proteinase, (approximately 0.01 per cent); phosphate buffer of pH 7.5, *M*/15. The reaction was carried out at 30° under aerobic condition. A, bacterial proteinase; B, trypsin.

Comparison of Digestion of Oxidized and Reduced Yeast Cytochrome c's by Bacterial Proteinase—It has been reported that oxidized yeast cytochrome c is digested more rapidly by bacterial proteinase than the reduced form, though the latter is also digested fairly rapidly by the proteinase under aerobic condition (*1*). Re-examination of this phenomenon shown that the raduced form is digested by the proteinase in a complicated way, for there is an inflection point in this reaction as shown in Fig. 3. The percentage digestion increases almost in parallel with the decrease of the optical density at 550 mμ.

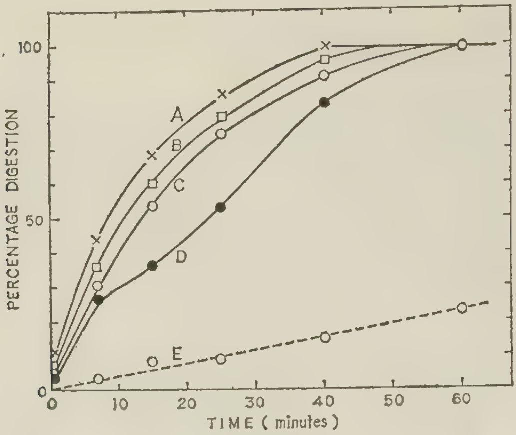

Fig. 2. Comparison of digestions of yeast and bovine cytochrome c by trypsin.

Reaction mixture as follows: Cytochrome c, 0.07 per cent; trypsin, 0.01 per cent; phosphate buffer of pH 7.5, $M/15$. Reaction was carried out at 30° under aerobic condition. A, oxidized, boiled yeast cytochrome c (oxidized form, 97 per cent); B, oxidized, TCA-treated yeast cytochrome c (oxidized form, 97 per cent); C, oxidized, native yeast cytochrome c (oxidized form, 95 per cent); D, reduced, native yeast cytochrome c (reduced form, 85 per cent); E, oxidized, native bovine cytochrome c (oxidized form, 95 per cent).

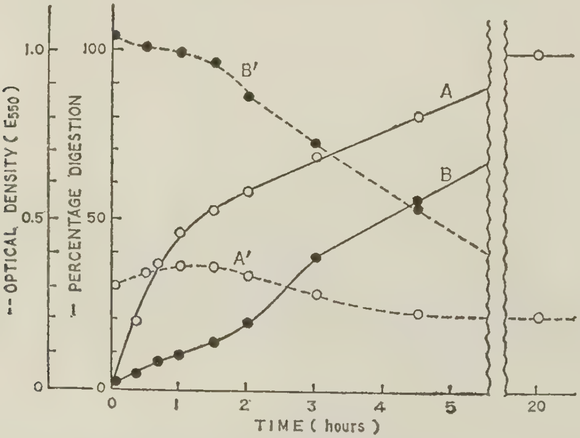

Fig. 3. Comparison of digestions on oxidized and reduced yeast cytochrome c by bacterial proteinase.

Native yeast cytochrome c was used. The oxidized sample consisted of 85 per cent of the oxidized form and 15 per cent of the reduced form, and the reduced sample consisted of 85 per cent of the reduced form and 15 per cent of the oxidized form. Reaction mixture as follows: Cytochrome c, 0.06 per cent; bacterial proteinase, 0.005 per cent; phosphate buffer of pH 7.5, $M/15$. Reaction was carried out at 15° underaerobic conditions. Broken lines show the change in intensity of the α-absorption peak of cytochrome c. Without addition of the proteinase, the intensity was scarcely influenced in either form during the incubation. A and A′, oxidized yeast cytochrome c; B and B′, reduced yeast cytochrome c.

The absorption spectra befor and after digestion of oxidized and reduced yeast cytochrome c's are shown in Fig. 4. Oxidized, native yeast cytochrome c shows absorption maxima at 530,410 and 358 mμ. Reduced and oxidized forms are finally both digested by bacterial proteinase to an oxidized form, as shown in Fig. 4. After digestion the γ- and δ-absorption peaks shift to 405 and 353 mμ, respectively, and the former increases in intensity. When reduced by sodium dithionite, the digested cytochrome c shows α- and β- absorption maxima in the same regions as the native form, but the γ-absorption maximum shifts from 415 to 417 mμ with an accompanying decrease in intensity.

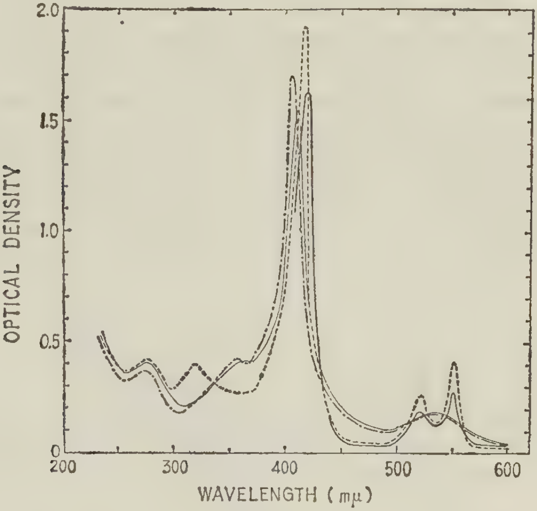

Fig. 4. Effect of bacterial proteinase-digestion on absorption spectra of yeast cytochrome c.

Oxidized, native yeast cytochrome c was digested by bacterial proteinase for 20 hours under the same conditions as in Fig. 3, except that the concentration of cytochrome c was 0.025 per cent. ——, native yeast cotochrome c in oxidized form; ----, native yeast cytochrome c in reduced form (prepared by sodium dithionite-reduction followed by dialysis against Na_2HPO_4, 0.1 *M*) —·—, yeast cytochrome c digested by bacterial proteinase; ——, digested yeast cytochrome c in its reduced from (measured under anaerobic conditions because of its remarkable autoxidizability).

Digestion of Reduced Yeast Cytochrome c by Bacterial Proteinase under Aerobic and Anaerobic Conditions—When either reduced or oxidized yeast cytochrome c, prepared by the method mentioned above, is dialyzed, the solution obtained contains a little of the other form. Thus, "reduced cytochrome c" used in these experiments is contaminated with about 5–15 per cent of the oxidized form, and "oxidized cytochrome c" with about 5–15 per

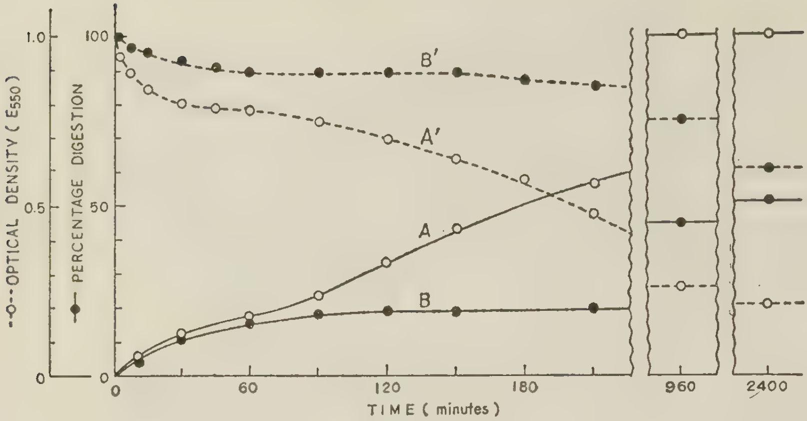

FIG. 5. Digestions of reduced, native yeast cytochrome c by bacterial proteinase under aerobic and anaerobic conditions.

Reaction mixture as follow: Reduced, native yeast cytochrome c, 0.05 per cent: bacterial proteinase, 0.005 per cent; phosphate buffer of pH 7.2, $M/15$. Reactions were carried out undr aerobic and anaerobic conditions at 15°. The reduced sample consisted of 85 per cent of the reduced form and 15 percent of the oxidized form. A and A′, under aerobic conditions; B and B′, under anaerobic conditions.

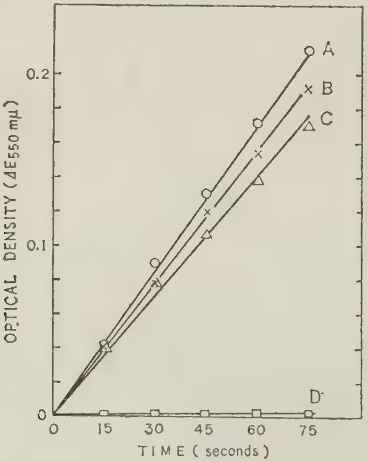

FIG. 6. Reduction of modified and digested yeast cytochrome c by yeast lactic dehydrogenase.

Reaction mixture as follows: 0.072 per cent of yeast cytochrome c in 0.1 M phosphate buffer of pH 7.2, 3.0 ml.; yeast lactic dehydrogenase, 0.2 ml.; 0.05 M sodium lactate, 0.5 ml. The reaction was started by adding the enzyme solution and the incubation was made at a room temperature under anaerobic conditions. A, oxidized, native yeast cytochrome c; B, oxidized yeast cytochrome c modified by TCA-treatment; C, oxidized, boiled yeast cytochrome c; D, oxidized yeast cytochrome c digested by bacterial proteinase.

cent of the reduced form. As a result it is found that during the first 1.5 hours in anaerobic conditions, reduced cytochrome c is digested by bacterial proteinase at almost the same rate as in aerobic conditions (Fig. 5). However, after this period under anaerobic conditions digestion ceases, while under aerobic conditions it continues. Casein is digested at a same rate under both anaerobic and aerobic conditions. This shows that reduced, native yeast cytochrome c when contaminated with its oxidized form is digested by the proteinase, but not when it is entirely the reduced form. However, as with TCA-treated and boiled preparations, during digestion by the bacterial proteinase, yeast cytochrome c increase in autoxidizability. In the presence of oxidized cytochrome c which has been partially digested by the proteinase and then boiled for 10 minutes to inactivate the proteinase, oxidation of reduced yeast cytochrome c is acccerelated. Therefore, it seems likely that native yeast cytochrome c in its reduced form is oxized by the digested cytochrome c and that then the resulting oxidized form is digested by the proteinase at the increased rate shown in curve A of Fig. 5.

Comparison on Reduction of Native, Modified and Digested Yeast Cytochrome c's by Yeast Lactic Dehydrogenase—As described in the previous paper (*1*), native yeast cytochrome c appears in fraction IV when chromatographed on Amberlite XE-64, but when modified by TCA-treatment or boiling, although the spectra of the oxidized and reduced forms are practically unchanged, it appears in fraction I. These modified forms of yeast cytochrome c can be reduced by lactate in the presence of yeast lactic dehydrogenase at almost the same initial rate as the native form, as shown in Fig. 6. However, the yeast cytochrome c after digestion with bacterial proteinase is not reduced even under anaerobic condition.

DISCUSSION

During purification by chromatography on an Amberlite XE-64 column, yeast cytochrome c in its oxidized form is separated into a number of fractions. In the previous paper (*1*), it was shown that it could be separated into four fractions I to IV. These four fractions have almost the same absorption spectrum in their oxidized and reduced forms, and are reduced to about the same extent and at almost the same initial rate by lactate in the presence of the yeast lactic dehydrogenase. Fraction IV is identified with native yeast cytochrome c, since it is the most resistant of the four fractions to digestion by bacterial proteinase, and since the milder is the purification procedure, the more is the cytochrome c content of the fraction IV. When native yeast cytochrome c is treated with TCA or is boiled, it all appears in fraction I and it becomes more susceptible to proteinase digestion than the native form. This shows that destruction of the secondary structure of yeast cytochrom c by these treatments scarcely influences to its absorption spectrum or specificity as an acceptor for yeast lactic dehydrogenase. However, Henderson and Rawlinson (*5*) have found that a modified form of mammalian cytochrome c has a higher oxidation-reduction protential than the native form.

On the other hand, when native yeast cytochrome c is digested by bacterial proteinase or trypsin, the resulting compound has an essentially different spectrum from those of the native or modified form and is not reduced by yeast lactic dehydrogenase. These facts suggest that the hememoiety and the amino acid sequence of the protein-moiety of yeast cytochrome c but almost not the secondary structure of the protein-moiety, affects its absorption spectrum and its specificity to the dehydrogenase.

Kunitz ahd McDonald (*6*) have found that crystalline hexokinase purified from baker's yeast is easily digested and inactivated by trypsin. However, addition of glucose prevents its inactivation. The protective effects of its substrate were in the following order; glucose>fructose> mannose. On the other hand, α-amylase (*7*), triose-phosphate dehydrogenase (*8*), etc. have a certain secondary structure which appears not to be destroyed by bacterial proteinase and trypsin digestion even in the absence of their substrate. However, the presence of their substrates and co-enzyme protects these enzymes from denaturation and inactivation by chemical reagents, such as urea, or from destruction by heating or acidification. These treatment are thought to destroy the secondary structure of a globular protein, perhaps destroying hydrogen bonds. At any rate, this protection by the substrate or co-enzyme is probably due to a change in the enyme protein as shown in equation A:

$$\text{Static state} \xrightleftharpoons[\substack{\text{in the absence}\\ \text{of its substrated and co-cnzyme}}]{\text{in the presence}} \text{Active state} \qquad \text{(A)}$$

In the case of native yeast cytochrome c, equation A may be divided further into the following two equations, B and C:

$$\text{Fe}^{+++}\text{ in heme-moiety} \xrightleftharpoons[\text{oxidized}]{\text{reduced}} \text{Fe}^{++}\text{ in heme-moiety} \qquad \text{(B)}$$

$$\begin{array}{c}\text{Proteinase-susceptible}\\ \text{state of protein-moiety}\end{array} \xrightleftharpoons[\text{oxidized}]{\text{reduced}} \begin{array}{c}\text{Proteinase-resistant}\\ \text{state of protein-moiety}\end{array} \qquad \text{(C)}$$

where B expresses oxidation and reduction of the iron in the heme-moiety of native yeast cytochrome c and C expresses a change in the protein-moiety. It seems likely that native yeast cotochrome c is more similar to hexokinase with regard to the effect of proteinase digestion than to the other enzymes mentioned above, and C is closely associated with reaction B which in turn is coupled with the electron transport system in respiring yeast cells. In the modified yeast cytochrome c, reaction C is possibly blocked by destruction of the secondary structure of its protein-moiety, while reaction B is unaffected.

SUMMARY

1. Yeast cytochrome c was modified by TCA-treatment and by boiling. These modified forms of cytochrome c are more susceptible to digestion by trypsin than the native form, in the following order: Oxidized, boiled yeast cytochrome c>Oxidized, TCA-treated yeast cytochrome c Oxidized, native yeast cytochrome c>Reduced, native yeast cytochrome c>Oxidized, native bovine cytochrome c.

2. Native yeast cytochrome c in its reduced form is scarcely digested by bacterial proteinage and trypsin, while in its oxidized form is rapidly digested. The cytochrome c in its rednced form is digested after being oxidized by the catalytic action of the digested cytochrome c under aerobic, but not appreciably under anaerobic conditions.

3. Yeast cytochrome c modified by TCA-treatment or boiling shows almost the same absorption spectra as that of the native form, but when digested by bacterial proteinase and trypsin it shows spectra especially of the γ- and δ-bands which are different from those of the native form.

4. Modified yeast cytochrome c is reduced by yeast lactic dehydrogenase in the presence of lactate at almost the same initial rate as the native form, but the digested form is not reduced even under anaerobic conditions.

From these facts, the relation between the electron transfer by yeast cytochrome c and the configuration of its protein-moiety is discussed.

We would like to express our thanks to Messrs. T. Higashi, T. Yamanaka, and J. Yamashita for their help and discussion during this investigation, and to Mr. K. Fujii (Oriental Yeast Co., Ltd., Suita, Osaka, Japan) for his advices and for his supplying us with baker's yeast.

REFERENCES

(*1*) Nozaki, M., Yamanaka, T., Horio, T. and Okunuki, K., *J. Biochem.* **44**, 453 (1957)
(*2*) Hagihara, B., Horio, T., Yamashita, J., Nozaki, M., and Okunuki, K., *Nature*, **178**, 629 (1956)
(*3*) Hagihara, B., Morikawa, I. Sekuzu, I., Horio, T., and Okunuki, K., *Nature*, **178**, 630 (1956)
(*4*) Yamanaka, T., Horio, T., and Okunuki, K., *J. Biochem.* **45**, 291 (158)
(*5*) Henderson, W., and Rawlinson, W.A., *Nature*, **177**, 1180 (1946)
(*6*) Kunitz, M. and McDonald, M.R., *J. Gen. Physiol.*, **29**, 393 (1946)
(*7*) Yamanaka, T., Higashi, T., Horio, T., and Okunuki, K., *J. Biochem.* **44**, 637 (1957)
(*8*) Nozaki, M., Sekuzu, I., Hagihara, B., Yonetani, T., Nakai, M., and Okunuki, K., *J. Biochem.* **44**, 595 (1957)

The Journal of Biochemistry, Vol. 45, No. 10, 1958

HALOPHILIC ALDOSE DEHYDROGENASE

I. SUBSTRATE SPECIFICITY AND SOME PROPERTIES OF THE ENZYME*

By MASAYUKI KATAGIRI, HIROO MAENO**
AND KATSUJI HORI

(*From the Chemical Institute, Faculty of Science, Nagoya University, Nagoya*)

(Received for publication, January 20, 1958)

Previous investigations from this laboratory (*1*, *2*) on glucose dehydrogenase of halophilic *Pseudomonas No. 101* (*3*) have indicated that the ultrasonic or the waterlysed extracts, having typical halophilic nature in respect both to stability and to activity, were also active on D-xylose and lactose.

Evidence presented in this paper suggested that partially purified particlefree extracts oxidized hexoses, pentoses and disaccharides in the presence of 2, 6-dichlorophenolindophenol, under a rule which, so far the configurations concerned, resembled to the Bean-Hassid's carbohydrate oxidase (*4*), although our preparation showed a wider specificity in respect to the carbon numbers of the aldose. Furthermore, the mechanism of aldose oxidation of our enzyme seemed not to be the of a notatin like enzyme since no oxygen uptake was observed with curde with crude extracts in the presece of substrate. This enzyme, therefore, will be referred in this paper to halophilic aldose dehydrogenase.

EXPERIMENTAL

Reagents—All substances used were obtained commercially except purified I-galactose, which was generously furnished by Dr. C. Araki of Kyoto University of Industrial Arts and Textile Fibers: D-arabinose, 2-deoxy-D-glucose, 2-deoxy-D-ribose, dihydroxyacetone, glucuronic acid, D, L-glyceraldehyde, glycol aldehyde, glycolic acid, D-lyxose and I-sorbse were from California Foundation for Biochemicol Research (U.S.A) and other substrates were from Wako Pure Chemical Industries (Japan). Each sugar other than 2-deoxy-D-ribose was further tested paperchromatographically using phenol-water (4 : 1) as a solvent system. The tests proved all sugars to be pure except 2-deoxy-D-glucose and D-galactose which were shown containing trace amounts of reducing substances having R_f value 0.31 and 0.27 respectively when 1.8 mg. of each sample was applied. The chemical pure grade sodium chloride was used throughout this work unless otherwise stated.

---

* This work was supported by a grant from the Scientific Research Fund of the Ministry of Education of Japan.

** Present adress: Department of Microbiology, School of Medicine, Gumma University, Maebashi.

Preparation of Cell Free Extracts—The strain used in this study was halophilic *Pseudomonas No. 101.* The cells were grown on about 0.5 cm. layer of medium in a large dish (1000 cm^2) containing 2 per cent of agar, 10 per cent of sodium chloride*, 1 per cent each of meat extract and polypeptone, and 0.5 per cent of $MgCl_2 \cdot 6H_2O$, since we found magnesium ions improved the enzyme production (as much as five folds). Although 10 per cent of sodium chloride was not enough for full production of the enzyme (*5*), the salt was concentrated by evaporation during the cultivation as the culure dishes were covered with sterilized papers. After 40 hour incubation at 30° the cells were harvested and washed twice with 10 per cent sodium chloride solution. Cell-free extracts were prepared by exposing the washed cells in 8 parts of distilled water, stirred in a Waring Blendor for 15 seconds at the maximal speed and the mixture was immediately centrifuged in cold at 20,000×*g* for 20 minutes (crude extracts). Crude extracts were further fractionated as noted later or immediately added by sodium choloride to 2 *M*.

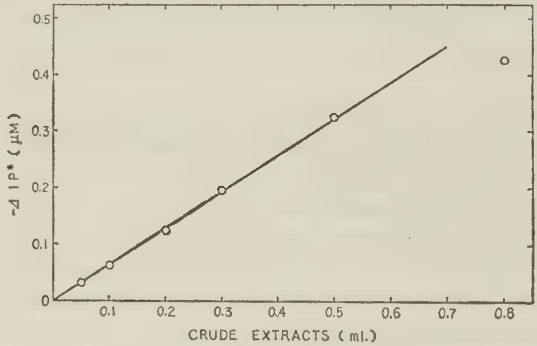

Fig. 1. The colorimetric assay of the aldose dehydrogenase. For the condition of the experiment, see the text.
* 2, 6-dichlorophenolindophenol.

Analytical Method—An enzyme unit was defined as the amount required to reduce 1 μ mole of 2, 6-dichorophenolindophelol per 6 minutes at 37° in the mixture of 400 μ moles of D-glucose, 0.5 μ mole of the dye and 200 μ moles of sodium phosphate buffer (pH 6.1) in 4 ml. of 2 *M* sodium chloride. The reaction mixture was extracted with 8 ml. of ethyl acetate, and the optical density of the extract was measured against a control omitted glucose with a filter (maximum absorption at 540 mμ). Under these conditions the rate of the reaction is linear and proportional to the enzyme concentration (Fig. 1). Specific activity was defind as the number of units per mg. protein determined by the colorimetric method of Folin and Ciocalteu modified by Lowry *et al.* (*6*). Aldonic acid was determined as hydroxamic acid by the method of Hestrin (*7*). Reducing sugars and pentoses were determined by the methods of Folin and Malmros (*8*), and Meijbaum (*9*), respectively.

---

* Japan Pharmacopoea grade of sodium chloride was used for culture medium exclusively. Although commercial table salt was less expensive, it contained some impurities which inhibited the enzyme production.

RESULTS

Purification of the Enzyme—All subsevuent steps in purification were conducted at 0–5° unless otherwise stated. To 100 ml. of fresh crude extracts were added 5 ml. of 1 M sodium acetate buffer (pH 5.0) and 21 g. of powdered ammonium sulfate. After the precipitate was discarded, 35 g. of ammonium sulfate per 100 ml. of the supernatant fluid were added. The resulting precipitate, collected by centrifugation, was dissolved in 20 ml. of 2 *M* sodium chloride solution without any buffer. Some insoluble materials were centrifuged off by a Spinco Model L ultracentrifuge at 100,000×*g* at the top for 30 minutes (Fraction I). Crude extracts usually contained approximately 1.7 units activity and 7 mg. of protein per ml.

TABLE I

Purification of Aldose Dehydrogenase

Enzyme fraction	Volume (*ml.*)	Units	Overall recovery (*per cent*)	Specific activity (*units per mg. protein*)
Crude extracts	145	254	100	257
Fraction I	31	223	88	1,210

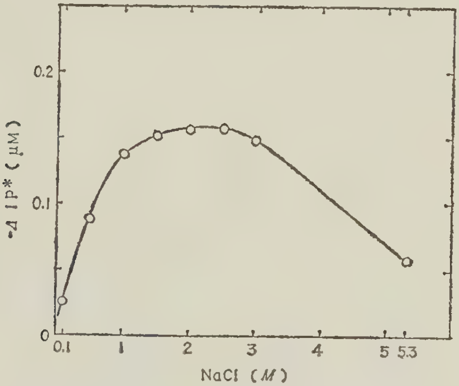

FIG. 2. Effects of sodium chloride concentration on the reaction rate. The experimental conditions were essentially identical with those of enzyme assay except the sodium chloride concentration. For each experiment, 0.02 ml. of the Fraction I was used.

* 2, 6-dichlorophenolindophenol.

Fraction I contained approximately 7 units of activity and 7 mg. of protein per ml. A typical result is shown in Table I. At—20°, Fraction I retained adequate activity for at least one month.

Properties of the Fraction I—The rate of the reaction was markedly dependent upon the ionic strength of the incubation mixture. At pH 6.1, the enzyme was most active at a sodium chloride concentration of about 2 *M* (Fig. 2) and even in saturated sodium chloride solution, the activity was almost 40 per cent of that observed in 2 *M* sodium chloride. The stability of the enzyme was also greatly dependent upon the salt concentration as is shown in Fig. 3.

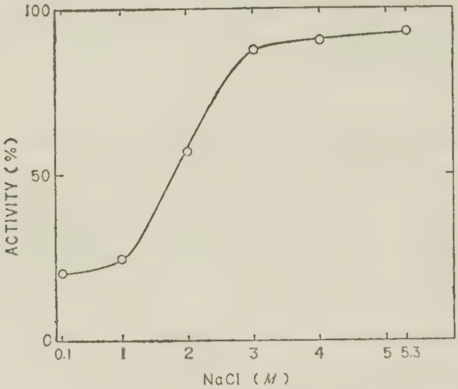

FIG. 3. Effect of sodium chloride concentration on the stability of the enzyme. After each concentration of 1 ml. sodium chloride solution containing 0.2 ml. of the Fraction I was kept at 54° for 4 minutes, the mixture was immediately cooled and the activity was measured at the standard assay condition.

TABLE II

Effect of pH on the Enzyme Reaction

	Activity ratio*		
Substrate	pH 5.1	pH 6.1	pH 7.1
D-Glucose	0.52	1.00	0.72
L-Arabinose	0.52	1.00	0.46
Maltose	0.33	0.65	0.43

For the condition of the experiment, assay method of the enzyme was applied using 0.02 ml. of Fraction I except the substitution of D-glucose by L-arabincse or maltose.

* Ratio to the value when D-glucose was a substrate at pH 6.1.

DPN or TPN reduction by D-glucose as a substrate was not observed spectrophotometrically even at high enzyme concentrations.

The optimal pH of the reaction was observed to be about 6, (Table II). When Fraction I was dialysed against 2 *M* sodium chloride at 4°, about 34 per cent of the original activity was lost after 3 days. This actiyity was restored, however, by the addition of even excess amount boiled crude extracts.

Substrate Specificity—Both crude extracts and Fraction I had activity on some of aldohexoses, aldopentoses, glyceraldehyde and disaccharides depending on their configurations. The activities of crude extracts and Fraction I on each sugar were summarized in Table III. Although each fraction was active on D, L-glyceraldehyde under the same conditions, the higher activity was found in the systems with lower salt concentrations, suggesting the activities, or at least a part of the activites, were due to another enzyme. Each fraction had essentially no activity on D-mannose, L-galactose, L-rhamnose, D-arabinose, D-lyxose, 2-deoxy-D-ribose, D-gluconic acid, D-glucuronic acid, D-glucosamine, D-galactosamine, sucrose, trehalose, D-mannitol, D-sorbitol, dulcitol, glycolaldehyde, glycerol, glyceric acid, acetic acid and formic acid.

TABLE III

Comparison of Rate of Dehydrogenation of Each Substrate wsth the Crude Extract and the Fraction I

Substrate	Crude extracts1)	Fraction I1)	Ratio2)
D-Glucose	1.00	1.00	1.00
D-Galactose	0.98	0.94	1.04
2-Deoxy-D-glucose	0.55	0.44	1.25
D-Ribose	0.85	0.87	0.98
L-Arabinose	1.00	0.98	1.02
D-Xylose	1.01	1.00	1.01
D, L-Glyceraldehyde3)	0.21	0.21	1.00
Lactose	0.85	0.87	0.98
Maltose	0.61	0.67	0.91
Cellobiose	0.89	0.91	0.98

Experimental conditions were iuentical with those for the assay of the enzyme except that 40 μ moles of each substrate was used instead of 400 μ moles of D-glucase.

1) Activity ratio to the value when D-glucose was a substrate.
2) Activity of Fraction I to that of crude extracts.
3) See the Text.

From the Lineweaver-Burk polt (*10*), the Michaelis constant

TABLE IV

The Michaelis Constant and the Maximal Velocity

Substrare	$K_m \times 10^3$	V_{max}*
D-Glucose	0.48	1.17
D-Galactose	1.33	1.53
D-Ribose	1.96	1.05
L-Arabinose	0.71	1.05
D-Xylose	0.77	1.17
Lactose	1.33	1.47
Maltose	10.0	1.29
Cellobiose	1.40	2.06

Experimental conditions were identical with those for the assay of the enzyme except 400 μ moles of D-glucose was substituted by various amounts of each sugar.

* Ratio to the value when the concentration of D-glucose was 0.1 *M*.

TABLE V

Stoichiometra of the Reaction, I

$-\Delta$D-Glucose (*μ mole*)	$-\Delta$ 2, 6-Dichlorophenolindophenol (*μ mole*)	Δ Hydroxamic acid (*μ mole*)
8.5	7.6	8.4

The reaction mixture contained initially D-glucose, 20 μ moles; sodium chloride 9 m moles; 2, 6-dichlorophenolindophenol 11 μ moles; sodium phosphate buffer at pH 6.1, 500 μ moles; and Fraction I, 2 ml. The final volume was 30 ml. The incubation was carried out at 37° for 60 minutes.

TABLE VI

Stoichometry of the Reaction, II

Substrate	$-\Delta$ Substrate (*mμ mole*)	Δ-2, 6-eichlorophenolindophenol (*mμ mole*)
D-Glucose1)	648	610
Lactose2)	491	457

1 μ mole of each substrate was incubated at 37° with 1 μ mole of 2, 6-dicholoropheholindophenol in 4 ml. of 2 *M* sodium chloride containing 0.2 ml. of the Fraction I.

1) Incubated for 4 minutes

2) Incubated for 60 minutes.

and maximal velocity of the reaction were estimated for each substrate (Table IV).

The Stoichiometry of the Reactions—When Fraction I was incubated with D-glucose in the presence of 2, 6-dichlorophenolindophenol, disappearance of the color was accompanied by the disappearance and the appearance of almost equal quantities of D-glucose and hydroxamic acid, respectively (Table V). The reaction mixture was further passed twice through Dowex 50 (hydrogen form) column to remove salts, chromatographed on paper using butanol-pyridine-water (6:4:3) as a solvent system (*11*) and a spot at R_f 0.16 which corresponded to D-gluconic acid was obtained.

The disaccharides seemed to be oxidized directly without preliminary hydrolysis, since, when lactose was used as a substrate, the decrease of reducing sugar was essentially equal to the amount of the consumption of the dye (Table VI).

DISCUSSION

In recent years, in addition to halophilic aldose dehydrogenase, several enzymes from halophilic (*1*, *10*, *12*, *13*) or halotolerant (*14*) microorganisms were found to require salt for their maximum activities. Some of these enzymes are irreversibly inactivated in the absence of salt. These evidences may suggest the existence of two halophilic phenomena, one is regarding to activity and the other to stability. However, as Maeno (*2*) indicated, the fact that halophilic aldose dehydrogenase had practically no activity in the absence of salts, was not due to the irreversible inactivation of the enzyme, although this enzyme is not quite stable in the absence of salt.

After partial purification of the enzyme, these halophilic natures regarding activity and stability, as well as other properties, were not changed.

The present observations also demonstrated that the halophilic aldose dehydrogenase has the stereospecificity with regard to the C-2 configuration of aldose, *i.e.*, the hydroxyl at C-2 should be to the right in the Fisher's projection formula, and is non-specific to that of C-3, C-4 and C-5 of aldohexose chains, and C-3 and C-4 configurations of aldopentose chains, respectively. Since this enzyme also catalyzes dehydrogenation of lactose, maltose and cellobiose but not L-rhammose and D-glucuronic acid, the hydroxyl of C-4 (hexose) may be substituted, but the primary hydroxyl group of C-6 (hexose) should be required. Although a similar enzyme from a red alga was described by Bean and Hassid (*4*), the latter enzyme is an oxidase and is speciffc for hexoses and disaccharides, while the aldose dehydrogenase attacked also pentoses.* The mechanism of aldose oxidation by our enzyme also seems to differ from that in liver, which involves a DPN specific glucose dehydrogenase linked to cytochrome (*15*). As Yamada *et al.* (*1*) and Maeno (*2*) reported, 2, 6-dichlorophenolindophenol and toluylene blue served as hydrogen acceptors from D-glucose, whereas methylene blue, pyocyanine and 2, 3, 5-triphenyltetrazolium chloride were inactive. They also demonstrated that the ultrasonic extracts of the halophilic bacteria *No. 101* were able to oxidize aldoses by atomospheric oxygen without the addition of hydrogen carriers. These facts may indicate that the mechanism of aldose oxidation in the halophilic bacterial extracts is similar to that in *Pseudomonas* reported by Wood and Schwerdt (*16*).

---

* We have had no chance to try aldotetroses as substrates.

SUMMARY

Halophilic aldose dehydrogenase from halophilic *Pseudomonas sp.* was partially purified by fractionation with ammonium sulfate. The particle free enzyme showed typical halophilic nature in respects both to stability and to activity.

The soluble enzyme, which appeared to be specific with regard to the C-2 configuration of the aldose chain (*i.e.*, the hydroxyl should be to the right), catalysed dehydrogenation of aldohexoses, aldopentoses and disaccharides in the presence of 2,6-dichlorophenolindophenol.

REFERENCES

(*1*) Yamada, T., and Asano, A., *J. Biochem.*, **41**, 639 (1954)
(*2*) Maeno, H., *J. Biochem.*, **44**, 99 (1957)
(3) Masui, M., personal communications.
(*4*) Bern, R.C., and Hassid, W.Z., *J. Biol. Chem.*, **218**, 425 (1956)
(*5*) Maeno, H., Shirakata, H. and Katagiri, M., unpublished results.
(*6*) Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., *J. Biol. Chem.*, **193**, 265 (1951)
(*7*) Hestrin, S., *J. Biol. Chem.*, **180**, 249 (1949)
(*8*) Folin, O., and Malmros, H., *J. Biol. Chem.*, **83**, 115 (1929)
(*9*) Meijibaum, W., *Z. physiol. Chem.*, **258**, 117 (1939)
(*10*) Lineweaver, H. and Burk, D., *J. Am. Chem. Soc.* **56**, 658 (1934)
(*11*) Koepsell, H.J., Stodola, F.H. and Sharpe, E.S., *J. Am. Chem. Soc.*, **74**, 5142 (1952)
(*12*) Baxter, R.M. and Gibbons, N.E., *Can. J. Biochem. Physiol.*, **32**, 206 (1954)
(*13*) Baxter, R.M. and Gibbons, N.E., *Can. J. Microbiol.*, **3**, 461 (1957)
(*14*) Baxter, R.M. and Gibbons, N.E., *Can. J. Microbiol.*, **2**, 599 (1956)
(*15*) Eichel, B. and Wainio, W.W., *J. Biol. Chem.*, **175**, 155 (1948)
(*16*) Wood, W.A. and Schwerdt, R.F., *J. Biol. Chem.*, **201**, 501 (1953)

The Journal of Biochemistry, Vol. 45, No. 10, 1958

BIOSYNTHESIS OF HEME AND HEMEPROTEINS IN TISSUE CELLS

IRON INCORPORATION INTO HEME BY PREPARATIONS FROM RAT LIVER*

By SHIGEKI MINAKAMI

(From the Department of Physiological Chemistry and Nutrition, Faculty of Medicine, University of Tokyo)

(Received for publication, March 17, 1958)

Immature red cells, such as bone marrow cell and reticulocyte, as well as nucleated avian red cell, are known to have the ability to synthesize heme and hemoglobin *in vitro* and the mechanism of the process has been studied extensively by several investigators. The outline of porphyrin biosynthesis was established by Shemin and his collaborators (*2*) using duck erythrocyte and by Rimington and his collaborators (*3*) who used chicken red cell. The mechanism of iron insertion into protoporphyrin was also studied using bone marrow cell (*4*), reticulocyte (*5*) and avian erythrocyte (*6*, *7*).

Few studies have, however, been made about the heme synthesis in organs other than hematopoietic system. Every aerobic cell has hemeproteins for their respiration and has to synthesize their prosthetic group *in situ*. Theorell and his collaboratos investigated the incorporation of radioactive iron into various hemeproteins of tissues *in vivo* and suggested the independent synthesis of hemeproteins in the cells (*8*). The capability to catalyze some steps of porphyric synthesis was demonstrated in liver homogenate (*9*, *10*).

The last step of heme synthesis, the insertion of iron into protoporphyrin was stutied so far little in cells other than hematopoietic system. It has been known that insertion of iron into protoporphyrin does not occur spontaneously and hematin iron does not exchange with inorganic iron (*11*). It may be of interest to investigate the system which catalyze the incorporation of raioiron into heme, and to characeterize the proteins to which the synthesized radioactive heme attach.

The present paper deals with the incorporation of Fe^{59} into heme *in vitro* by the homogenate, as well as by the particulate components and the cholate extract of rat liver. The active principle to incorporate radioactive iron into heme was further purified by ammonium sulfate fractionation.

---

* The preliminary reports of this paper have already been published (*1*).

EXPERIMENTAL

Incorporation of Iron into Heme by Liver Homogenate—The activity to incorporate radioactive iron into heme was examined in the homogenate of rat liver. The tissue was homogenized with 1/15 *M* phosphate buffer pH 7.0–7.4 using a Potter-Elvehjem homogenizer. The homogenate was incubated with radioactive ferric chloride-Fe^{59} (0.1–0.5 μc, 0.1–0.5 μg. iron) at 37°, shaking in manometric vessels.

After incubation, the sample was precipitated with trichloroacetic acid and centrifuged. The precipitate was extracted with a mixture of acetone and hydrochloric acid (10 ml. of 20 per cent hydrochloric acid in 1 liter of acetone) under homogenization in a glass homogenizer. The extract was concentrated, mixed with 1 ml. of chloroform and hematin was taken up in the chloroform layer by the addition of water. After washing with several volumes of water, the chloroform was evaporated. Then the pigment was separated chromatographically on a celite column (*12*): The dry residue was dissolved in a drop of pyridine and 0.5 ml. of chloroform and placed on the column of celite (Hyflo-Super-Cel) which had been previously wetted with 8 per cent acetic acid-chloroform (*v/v*) and eluted with the same solvent. The red brown portion of the effluent was collected for the estimation of radioactivity and heme content. The contaminating radioactive iron other than heme could be eliminated by this procedure. Radioactivity was determined by well-type scintillation-counter (Scientific Research Institute, Tokyo) and heme content of the sample was determined photometrically at 560 mμ. Radioactivity values were corrected on the basis of the recovery of heme in the procedure and expressed as total radioactivities of heme extracted from the sample.

TABLE I

Recrystallization of the Radioactive Hemin

	Radioactivity (*c.p.m.*)	Hemin (*mg.*)	Specific activity (*c.p.m./mg.*)
The chloroform extract	6.280	300 (carrier added)	2.09
1st crystallization	2.380	120	1.9
2nd crystalization	1.680	100	1.68
3rd crystallization	1.430	90	1.60

For further varification of the fact that radioactive iron was actually incorporated into heme, hemin was crystallized from the chloroform extract by the method of Fischer (*13*) after addition of a small amount of hemin as carrier. Specific activity became constant after repreated recrystallization (Table I). The identity of the radioactive iron complex with heme was also proved chromatographically: The peak of radioactivity and optical density of protoheme in the fractions of the effluent coincided well with each other and ratio of the two remained constant throughout the fractionation (Fig. 1).

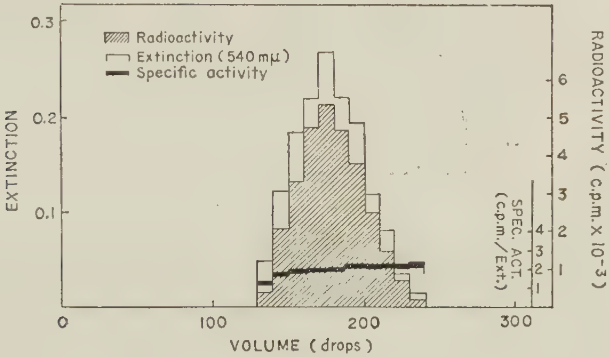

FIG. 1. Chromatography of the radioactive hemin. Hyflo-Super-Cel column (4.8×0.7 cm.) wetted with 8 per cent acetic acide chloroform (*v*/*v*) was used and eluted with the same solvent.

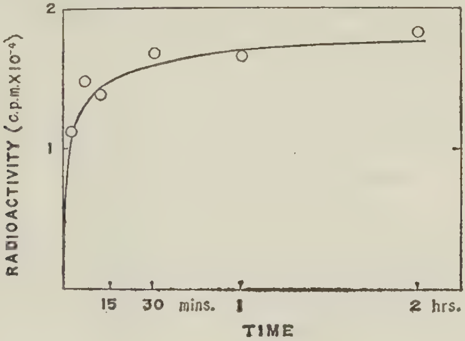

FIG. 2. Incorporation of radioiron into heme by rat liver homogenate. The homogenate of rat liver (about 0.4 g.) was incubated with Fe^{59} (about 2×10^5 c.p.m.) at 37°.

Almost instant incorporation of radioactive iron into heme was observed and saturation occurred within several minutes as shown in Fig. 2. After 1 hour, another portion of radioactive iron was added to the incubation sample. Then incorporation began again (Fig. 3). This experiment indicates that the saturation is not due to the exhaustion of any essential factor in the homogenate but due to the inaccessibility of radioactive iron. Trapping of iron by natural chelating agents may be suggested.

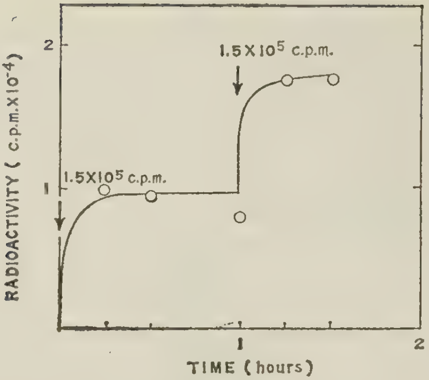

Fig. 3. Incorporation of radioiron into heme by the rat liver homogenate by the repeated additions ot radioiron. The rat liver homogenate (0.5 g. wet weight) was incucated with Fe^{59} (about 1.5×10^5 c.p.m.) at 37°. The second addition of radioiron was carried out 1 hour after the addition of the first.

Similar experiments were carried out using the homogenates of various tissues. The homogenates of various organs examined, *i.e.* kidney, brain, spleen, heart muscle and voluntary muscle had the similar activity to incorporate radioiron into heme (Table II).

Table II

Incorporation of Radioactive Iron into Heme by the Homogenate of various Organs (c.p.m./g. wet weight)

	Liver	Spleen	Kidney	Brain	Heart	Muscle
No. 1	2,340	4,320	3,230	1,550	4,500	300
No. 2	1.040	420	5,620	1,430	3,940	460

The homogenates were incubated with Fe^{59} (No. 1, 6×10^4; No. 2, 7.5×10^4 *c.p.m.*) at 37° for 2 hours. Total counts of the chloroform extract were estimated.

Distribution of Radioactivity in Particulate Fractions after the Incubation of Homogenate—After incubation with radioactive iron, the homogenate was fractionated by the fractional centrifugation method of Hogeboom (*14*). The homogenate was layered carefully over 0.34 *M* sucrose and centrifuged 10 minutes at 2,000 r.p.m. (nuclear fraction). The supernatant was centrifuged for 10 minutes at 5,000×*g* (mitochondrial fraction). Heme was extracted from each fraction and purified as before. The radioactivity in heme was the highest in the mitochondrial fraction and the lowest in the combined fraction of supernatant and microsome (Table III).

Table III

Distribution of the Radioactive Heme in the Subcellular Components of Rat Liver after Incubation

	Nucleus	Mitochondria	Supernatant+microsome
Radioactivity (*c.p.m.*)	4,520	14,900	4,720

The homogenate prepared from 2 g. wet weight of rat liver was incubated with Fe^{59} (about 3×10^5 c.p.m.) at 37° for 1 hour. centrifugation.

Distribution of the Synthesized Radioactive Hemeprotein in Ammonium Sulfate Fractions—After incubation with radioactive iron, the mitochondria was subjected to cholate extraction and ammonium sulfate fractionation to characterize the nature of the hemeprotein synthesized. The method was essentially the same with that of purification of cytochrome b from pig heart particle by Stotz (*15*). The mitochondria was extracted by 1 per cent sodium cholate in 1/15 *M* phosphate buffer pH 7.2 using a glass homogenizer cooling in an ice bath. To the supernatant obtained by centrifugation at 1,000×*g* for 20 minutes, was added fine powder of ammonium sulfate to obtain the desired saturations. The fractirns of 0–35, 35-50 and 50–75 per cent ammonium saturations were obtained by centrifugation at 10,000×*g* for 15 minutes. Heme was extracted from each fraction and purified.

Radioactivity was high in the fraction of 35–50 per cent ammonium sulfate saturation, as shown in Table IV. This fraction contained tained several heme proteins and showed reddish appearance.

Table IV

Distribution of the Radioactive Heme Ptotein in the Ammonium Sulfate Fraction of the Cholate Extract after Incubation

	Ammonium sulfate saturation (%)		
	0–35	35–50	50–75
Radioactivity (*c.p.m.*)	2,900	10,500	1,220

The mitochondria prepared from 4 g. wet weight of rat liver was incubated with Fe^{59} (about 5×10^5 c.p.m.) at 37° for 1 hour. The cholate extract of the sample was fractionated using ammonium sulfate.

The extraction and purification of cytochrome c from the mitochondria was carried out to examine whether radioactive iron was incorporated into the heme of cytochrome c. The mitochondria incubated with radioiron was extracted with 0.1 *N* sulfuric acid, neutralized and diluted about three fold. To the solution about 0.1 mg. of cotochrome c was added as a carrier and the solution was treated by Amberlite IRC 50. Cytochrome c absorbed on the resin was eluted with dilute ammonia water. The eluent completely lacked of radioactivity.

Incorporation of Iron into Heme by Cellular Components—Rat liver was perfused thoroughly with isotonic saline and 0.25 M sucrose solution to eliminate as possible the contamination of red cell to the nuclear fraction. The fractional centrifugation method of Hogeboom (*14*) was used for the fraction of cellular components. The microsome was separated from the supernatant by centrifugation at $20.000 \times g$ for 1 hour. The particles were suspended in phosphate buffer pH 7.2 and incubated with radioactive iron shaking at 37° as before. After extraction and purification, the radioactivity of heme was determined.

TABLE V

Activities of the Subcellular Components to Incorporate Radioactive Iron into Heme

	Nucleus	Mitochondria	Microsome	Supernatant
Radioactivity (*c.p.m.*)	1,250	7,080	2,380	2,260

The homogenate prepared from 2 g. wet weight was fractionated by centrifugation. Each fraction was incubated with Fe^{59} (about 1.5×10^5 c.p.m.) at 37° for 1 hour.

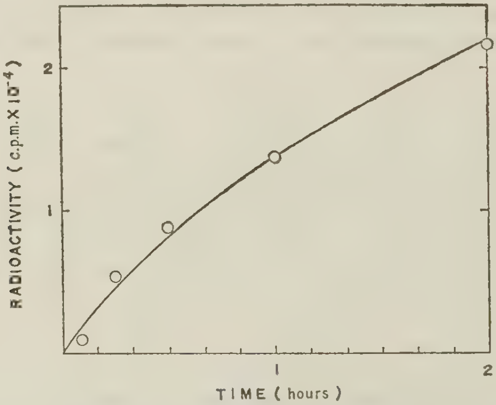

FIG. 4. Incorporation of radioiron into heme by mitochondria. The mitochondria prepared from 0.5 g. wet weight of rat liver was incubated with Fe^{59} (about 2×10^5 r.p.m.) at 37°.

Table V shows incoporation of Fe^{59} into heme by cellular components of rat liver. An exceedingly high radioactivity was observed in the mitochondrial fraction. The activity to incorporate radioactive iron into heme was firmy bound to cellular components and could not be washed out with saline or buffers.

In contrast to the experiment using the homogenate, the time course of the iron incorporation into heme by mitochondria was almost linear for several hours (Fig. 4).

Purification of the System to Incorporate Radioactive Iron into Heme—The system to incorporate radioactive iron into heme could be solubilized from mitochondria by bufferized sodium cholate solution (1 per cent sodium cholate in 1/15 *M* phosphate buffer pH 7.2) using a glass homogenizer placed in an ice bath. The supernatant obtained by centrifugation at 10,000×*g* for 15 minutes was used for the experiment. This was incubated with radioactive iron at 37°.

The cholate extract was further purified by ammonium sulfate fractionation. Fine powder of ammonium sulfate was added to the extract to obtain the desired saturation. The fractions of 0–35, 35–50 and 50-75 per cent ammonium sulfate satuations were precipitated by centrifugation and dissolved in 1/15 *M* phosphate buffer. Then the fractions were dialyzed against distilled water overnight. Method of incubation and the estimation of radioactive hemin were as described before.

Table VI

Activities in the Ammonium Sulfate Fractions of the Cholate Extract to Incorporate Radioiron Heme into

	Ammonium sulfate saturation (%)			
	0–30	35–50	50–75	75–
Protein contents (*mg.*)	118	220	13	—
Radioactivity (*c.p.m.*)	1,820	1,750	19,100	150

The mitochondria prepared from 5 g. wet weight of rat liver was extracted using cholate. The extract was fractionated by ammonium sulfate precipitation and dialyzed against distilled water. The dialyzate was incubated with F^{59} (about 5×10^5 c.p.m.) and cysteine (1 μ mole) at 37° for 1 hour.

Most of the activity to incorporate radioactive iron into heme existed in the fraction precipitated between 50 and 75 per cent ammonium sulfate saturation (Table VI). The protein content of this fraction as estimated by turbidimetry using sulfosalicylic acid (*16*) was proved to be small as compared with those of other fractions. The cholate extract was thus purified more than twenty times by ammonium sulfate fractionation. Addition of any other fraction did not cause any further increase in ihe incorporation.

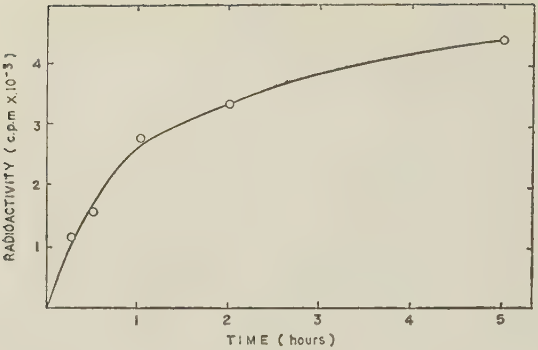

Fig. 5. Incorporation of radioiron into heme by the cholate extract of rat mitochondria. The cholate extract prepared from 0.5 g. wet weight of rat liver was incubated with Fe^{59} (about 2×10^5 c.p.m.) and cysteine (1 μ mole) at 37°.

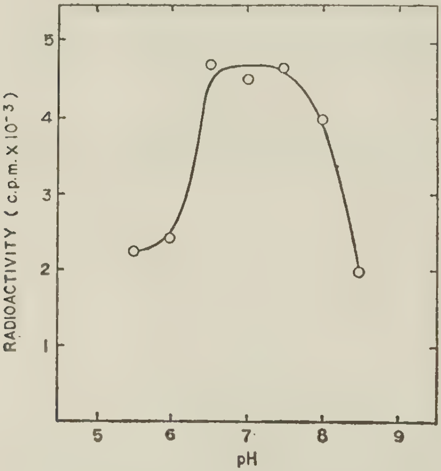

Fig. 6. Influence of pH on activity of the cholate extract. The cnolate extract prepared from 0.5 g. wet weight of rat liver was incubated Fe^{59} (about 2×10^5 c.p.m.) and cysteine (1 μ mole) at 37° for 3 hours.

Some Properties of the Cholate Extract—The time course of iron incorporation into heme by the cholate extract was shown in Fig. 5. The incorporation increased almost linearly for several hours. The optimum range of pH for the incorporation lied at about 7 (Fig. 6), and the activity decreased as the pH of the solution became below 6 or above 8. The activity was abolished completely by heating in a boiling water bath for 30 seconds.

The cholate extract and the fractions of ammonium sulfate precipitation could be dialyzed without loss of activity. The addition of protoporphyrin (0.02 μmole) to the extract or the dialyzate did not cause any increase in the incorporation. Reducing agents such as cysteine was proved to have an accelerating activity (about two fold) (Table VII).

TABLE VII

Incorporation of Radioiron into Heme by the Cholate Extract

	Radioactivity (*c.p.m.*)
The cholate extract (non-dialyzed)	3,607
,, +protoporphyrin (0.02 μM)	3,920
,, +cysteine (1 μM)	7,160
The cholate extract (dialyzed)	4,200
,, +protoporphyrin (0.02 μM)	3.360
,, +cysteine (1 μM)	10,700

The cholate extract prepared from 1 g. wet weight of rat liver was incubated with Fe^{59} (about 2×10^5 c.p.m.) at 37° for 2 hours.

DISCUSSION

Whereas the biosynthesis of protein moiety of hemeproteins requires intact cell structure, the biosynthesis of heme-moiety is known to occur by broken cell preparations (*2*, *3*). The last step of heme formation, the insertion of iron into protoporphyrin ring has been observed by whole cell as well as broken cell preparation of nucleated red cells. This activity probably exists in the insoluble fraction of cell as shown by the experiment of Kasuga, Konno and Sakai (*4*). These authors fractionated the homogenate of bone marrow cells by the differential centrifugation method of Hogeboom and claimed that the activity exist in the nuclear fraction. It is conceivable, however, that the structure of bone marrow cell is quite different from those of the ordinary parenchymatous cells usually used for the preparation of cellular particles. The fractional centrifugation of the similar immature red cell, such as avian red cell, revealed that almost all the particulate components were found to be in the nuclear fraction of Hogeboom.

As indicated in the present investigation, incorporation of iron into heme was also observed by liver cells, though the amount of heme synthesized was small as compared with hematopoietic cells. The activity of the incorporation existed in the particulate fraction, especially in the mitochondrial fraction. The activity of each particulate fraction may roughly correspond to the ability to synthesize heme pigment in each particle. Cytochrome b_5 is known to exist in microsome, cytochrome b and other heme enzymes in mitochondria. As mitochondrial fraction is rich in heme enzymes, it is conceivable that this fraction is active in the biosynthesis of heme.

Bonnichsen and others (*17*) found "non-extractable" hemoglobin in the liver nucleus ann the incorporation of radioactive iron into this hemoglobin was high after intraperitoneal injection of Fe^{59}-labelled iron. The activity *in vitro* of liver nuclear fraction to incorporate radioactive iron into heme in the present experiments would be partly due to synthesis of so called non-extractable hemoglobin of Bonnichsen and others. It is known that liver and spleen are the main site of extramedullary hematopoiesis. Especially, the occurrence of extramedullary hematopoiesis was often obserbed in rat liver. It is possible that "non-extractable" hemoglobin of Bonnichsen and others be due to the extramedullary hematopoiesis. In some of the experiments, the present author has observed high rate of the incorporation by the nuclear fraction of liyer.

The activity to incorporate radioiron into heme was also observed in the homogenates of the other tissues as far as examined. The amounts of heme synthesized in different tissues could not, however, be calculated directly from radioactivity of the heme preparations, because of the difference in iron-pools available for heme synthesis. Nevertheless, the rate of the incorporation by the tissue homogenate was roughly proportional to the content of respiratory hemeproteins or the rate of oxygen consumption.

By incubation of the homogenate with radioactive iron, radioactivity was observed in the particulate fraction and could not be washed out. From this finding the fate of the heme synthesizec by insertion of iron into protoporphyrin ring could be discussed. The heme may be assumed to attatch to the protein of the particle simultaneously or soon after the insertion of iron. Ammonium sulfate fractionation of the cholate extract revealed that the hemeprotein synthesized by the incubation of mitochondria belonged to the same fraction with the known hemeproteins such as cytochrome b and catalase.

The system of the iron incorporation which was found in the mitochondrial fraction of rat liver, resembled to some extent to that of the nucleated erythrocyte of duck and chicken. The Tween 20 extract of duck red cell studied by Krueger and others (*18*) as well as the cholate extract of chicken red cell studied in the author's laboratory (*19*) have similar properties in common with the extract of liver mitochondria. The cholate extract of both chicken red cell and rat liver mitochondria, have the properties of protein nature and the activity was abolished completely by heattreatment or by extremely acidic or alkaline pH. The optimum range of pH lied about 7. Reducing agents such as cysteine or ascorbic acid prominently accelerated the incorporation.

The necessity of reducing state in the utilization of iron has been often pointed out. The absorption of iron by intestinal tract was known to occur in ferrous form. Neve and others (*20*) and Neilands and others (*21*) assumed that the insertion occurred to reduced porphyrins. However, as will be discussed later, the extract of chicken erythrocyte needed the addition of protoporphyrin for the incorporation of iron into heme and the possibility of protoporphyrin as an immediate precursor for heme was suggested. The mere addition of reducing agents such as cysteine may not cause reduction of protoporphyrin. The role of reducing agents is remained to be studied.

The main difference observed between the cholate extract of chicken red cell and that of liver mitochondria was that the former incorporated small proportion of radioiron into heme without the addition of protoporphyrin and prominent increase in the incorporation was observed by the addition of protoporphyrin, whereas the activity of the latter did not increase by the addition of protoporphyrin. The procedures of purification, such as ammonium sulfate fractionation and prolonged dialysis did not cause the loss of the activity in the case of the extract from liver mitochondria. The existence of some complex of protein-porphyrin in the fraction 50-75 per cent ammonium sulfate saturation may be suggested.

Recently Eriksen (*22*) have reached to the similar conclusion about hemoglobin synthesis in reticulocyte. He posturated two forms of protoporphyrin in erythrocyte, *i.e.* "free" and "bound" protoporphyrin. He though that "bound" protoporphyrin is formed from "free" protoporphyrin, and that the former is the immediate precursor to heme. He postulated that "bound" porphyrin was a complex of globin and protoporphyrin, and iron was incorporated into globin-protoporphyrin-complex directly. Thus the last step of hemoglobin synthesis was assumed to be the incorporation of iron.

The present investigation revealed the existence of another process which followed the iron incorporation, that is transfer of heme-group. The 50-75 per cent fraction did incorporate radioiron into heme and cause the formation of protein-heme complex. However, this complex is most presumably not a final product. Incubation of mitochondria with radioactive iron resulted in the formation of radioactive hemeproteins which could be precipitated by half saturation of ammonium sulfate. The heme synthesized by the catalyst of protein nature which belongs to the 50-75 per cent fraction, may be transfered to the apo-proteins of the heme-enzymes which belong to the 35-50 per cent ammonium sulfate saturation. Drabkin (*23*) suggested the possibility of heme transfer from cytochrome b_1 to cytochrome c in the adaptive formation of cytochrome c in yeast. In the present investigation, radioactivity was not observed in the heme of cytochrome c. The heme of cytochrome c is known to be attatched so firmly to the proteinmoiety that the transfer of heme is almost inconceivable. Transfer of heme may be restricted to the family of b-cytochromes, catalase or other protohemeproteins.

In the preceding report (*19*) about the cholate extract of avian erythrocyte to incorporate radioactive iron into heme, it was avoided to call the active principle an "enzyme", because there are certain possibilities that the active principle is some kind of protoporphyrin-globin complex which, after the insertion of iron, changes to hemoglobin and shows no catalytic property. The active principle in the present investigation which was found in the albumin-like fraction of the cholate extract from liver mitochondria, may properly be called an "enzyme" as the heme synthesized may be transfered from the "enzyme" to other proteins and able to catalyze the reaction further.

SUMMARY

1. Incorporation of radioactive iron into heme was observed by incubating the homogenate of rat liver. Heme was purified either by chromatography on celite column or by crystallization. The homogenates of other organs, such as kidney, brain, heart muscle, spleen and voluntary muscle had the similar activity.

2. The product of incorporation after incubating the homogenate with radioactive iron, was firmly bound to the particulate fractions. The heme in mitochondria had high rate of radioactivity. Radioactive hemeproteins were extracted by cholate after the incubation of mitochondria with radioiron. Radioactive heme existed in the fraction of 35-50 per cent ammonium sulfate saturation.

3. The activity to incorporate radioactive iron into heme was found in the particluate fraction of liver cell. The high activity was observed in mitochondrial fraction.

4. The active principle for the incorporation of iron into heme was solubilized from mitochondria with sodium cholate and purified by ammonium sulfate fractionation. The activity was found in the fraction of 50-75 per cent ammonium sulfate saturation.

5. The activity of the cholate extract had the optimum at about pH 7. The incorporation was accelerated by the addition of cystein. Addition of protoporphyrin did not cause any increase in the incorporation. The activity was totally abolished by heat-treatment.

6. The mechanism of hemeprotein synthesis in liver cell was discussed from the above findings.

The author expresses his cordial thanks to Prof. H. Yoshikawa and Prof. Y. Yoneyama for their kind guidance and encouragement in carrying out this work. Thanks are also due to Drs. Y. Kagawa, Y. Sugita, S. Morimoto and Mr. S. Tanaka of the author's laboratory and Mr. K. Titani, H. Ishikura and K. Takahashi of the Department of Chemistry, Faculty of Science for their encouraging discussions.

REFERENCES

(*1*) Minakami, S., Yoneyama, Y., and Yoshikawa, H., *Biochim. Biophys. Acta*, **28**, 447 (1958)
Minakami, S., and Yoneyama, Y., *J. Jap. Biochem. Soc.*, **30**, 211 (1958)
Minakami, S., Kagawa, S., Sugita, Y., Tanaka, S., Morimoto, S., and Yoneyama, Y., *J. Jap. Biochm. Soc.*, **30**, 285 (1958)

(*2*) Shemin, D., in "Porphyrin Biosynthesis and Metabolism" J. and A. Churchill, London p. 4. (1955)

(*3*) Rimington, C., International Symposium on Enzyme Chemistry, Tokyo, Oct. 1957.

(*4*) Kasuga, S., Konno, K., and Sakai, Y., *J. Jap. Biochm. Soc.*, **26**, 282 (1954)
Konno, K., *J. Jap. Biochem. Soc.*, **26**, 260 (1954)

(*5*) Yoshiba, A., Hattori, M., Nakao, K., Kagawa, Y. Morimoto, S., Minakami, S., and Yoneyama, Y., *J. Jap. Biochem. Soc.*, **30**, 297 (1958)

(*6*) Granick, S., *Fed Proc.*, **13**, 319 (1954)

(*7*) Jensen, W.N., Aschenbrucker, J., Cartwright, G.E., and Wintrobe, M.M., *J. Lab. Clin. Med.*, **42**, 833 (1953)

(*8*) Theorell, H., Beznak, M., Bonnichsen, R., Paul. K.G., and Akeson, A., *Acta Chem. Scand.*, **5**, 445 (1951)

(*9*) Gibson, K.D., in "Porphyrin Biosynthesis and Metabolism" J. and A. Churchill, London p. 27 (1955)

(*10*) Schwartz, S. and Ikeda, K., in "Porphyrin Biosynthesis and Metabolism" J. and A. Churchill, London, p. 209 (1955)

(*11*) Hahn, P.F., Bale, W.F., Hettig, R.A., and Whipple, G.H., *Science*, **92**, 131 (1940)

(*12*) Richmond, J.E., Altman, K.I., and Salomon, K., *J. Biol. Chem.*, **211**, 981 (1954)

(*13*) Fischer, H., *Organic Synthesis*, **21**, 53 (1941)

(*14*) Hogeboom, G.H., in "Methods in Enzymology" Academic Press, New York, Vol. **1**, p. 16 (1954)

(*15*) Stotz, E. in "Methods in Enzymology", Academic press, N.Y., Vol. **2**, p. 740 (1955)

(*16*) Layne, E. in "Methods in Enzymology", Academic press, N.Y., Vol. **3**, p. 447 (1957)

(*17*) Bonnichsen, R., Ehrenstein, G. Hevesy, G., and Schliack, J., *Acta Chem. Scand.*, **11**, 120 (1957)

(*18*) Krueger, R.C., Melnick, I., and Klein, J.R., *Arch. Biochem. Biophys.*, **46**, 302 (1956)

(*19*) Kagawa, Y., Minakami, S., Urata, G., Tanaka, S., Sugita, Y., Morimoto, S., and Yoneyama, Y. *J. Jap. Biochem. Soc.*, **30**, 292 (1958)

(*20*) Neve, R.A., Labbe, R.F., and Aldrich, R.A., *J. Am. Chem. Soc.*, **87**, 691 (1956)

(*21*) Neilands, J.B., International Symposium on Enzyme Chemistry, Tokyo, Oct., 1957.

(*22*) Eriksen, L., in "Porphyrin Biosynthesit and Metabolism" J. and A. Churchill, Lonnon p. 185 (1955)

(*23*) Drabkin, D.L., in "Porphyrin Bisoynthesis and metabolism" J. and A. Churchill, London, p. 96 (1955)

The Journal of Biochemistry, Vol. 45, No. 10, 1958

DIGESTION OF BAKER'S YEAST CYTOCHROME C IN OXIDIZED FORM BY BACTERIAL PROTEINASE UNDER AEROBIC AND ANAEROBIC CONDITIONS

As previously reproted (*1*, 2), native, crystalline cytochrome c from babker's yeast is in its oxidized form rapidly digested by bacterial proteinase and by trypsin, while it is hardly digested in its reduced form. It was noticed that the α- and β-absorption bands of cytochrome c increase in the intensity when bacterial proteinase is added to oxidized, native cytochrome c, and that soon after, these absorption bands decrease and disappear.

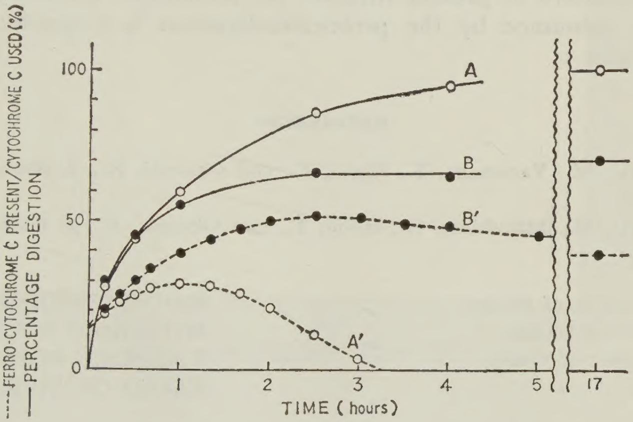

Fig. 1. Digestion of oxidized, native yeast cytochrome c by bacteria proteinase under aeroteinase under aerobicanaerobic conditions.

Reaction mixture as follows: Oxidized, native yeast cytochrome c, 0.13 per cent; bacterial proteinase, 0.005 per cent; phosphate buffer of pH 7.2, *M*/15. Reactions were made at 20° under air or nitrogen. "Ferro-cytochrome c present/cytochrome c used (per cent)" was calculated from extinctions at 550 mμ of sample at every experimental time and of ferricyanide-oxidized and dithionite-reduced samples at zero time. The percentage dipestion by the proteinase was calculated by the same way as in the previous paper (*1*, *2*).

A and A′, under aerobic conditions; B and B′, and, under anaerobic conditions.

This curious phenomenen was investigated in detail (Fig. 1). Under anaerobic conditions, oxidized yeast cytochrome c (containing 15 per cent of the reduced form) is more resistant to digestion by bacterial prateinase than it is under aerobic conditions, though there is no significant difference in the initial digestion-rate under the two conditions. On the other hand, the cytochrome c is reduced by addition of the proteinase under both anaerobic and aerobic conditions. Under aerobic conditions the proportion of the reduced form increases to 30 per cent, one hour after addition of the proteinase, but then decreases gradually until it disappears completely. Under anaerobic condition its proportion reaches 50 per cent after one hour and maintains the state even 17 hours after the begining of the reaction. Autodigested bacterial proteinase, having no proteolytic activity, does not reduce cytochrome c. This fact suggests that some substance is produced during the proteinase-digestion of cytochrome c which reduces the oxidized cytochrome c so that it cannot be digested. Under aerobic conditions oxidition of reduced cytochrome c is catalyzed by the digested cytochrome c which has an increased autoxidizability (*2*).

It is unknown at present whether the production of this cytochrome c-reducing substance by the proteinase-digestion is a specific property to cytochrome c.

REFERENCES

(*1*) Nozaki, M., Yamanaka, T., Horio, T., and Okunuki, K., *J. Biochem.* **44**, 453 (1957)

(*2*) Nozaki, M., Mizushima, H., Horio, T., and Okunuki, K., *J. Biochem.* in press

Department of Biology,
Faculty of Science,
University of Osaka,
Osaka.

HIROSHI MIZUSHIMA
MITSUHIRO NOZAKI
TAKEKAZU HORIO
KAZUO OKUNUKI

(Received for publication, April 15, 1958)